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13. ABSTRACT *(Maximum 200 Words)*

The purpose of this research project is to understand better the effect of dietary folate levels on the cellular pharmacology and toxicology of chemotherapeutic agents. The scope of the research involves *in vitro* studies with cell lines and *in vivo* assessments in rats of folate-chemotherapeutic drug interactions. Studies at a molecular level with human cells confirmed the model developed in rodent cells to explain the synergy between nutritional folate deficiency and alkylating agents. Cells expressing p53 activity exhibited a higher rate of mutation induction but were more sensitive to the toxic effects of alkylating agents than those lacking p53. Folate deficiency tended to reduce toxicity but increase mutation induction after alkylating agent treatment. Studies in rat liver confirmed that folate metabolism modulates glutathione levels. Studies in rats of the interaction of diet and an alkylating agent, cyclophosphamide, indicated that deaths were predicted by dose and diet, and by white blood count and renal function on Day 4 after chemotherapy. The combination of high doses of folic acid and chemotherapy caused renal damage, indicating that there may be an optimal amount of dietary folate to modulate toxicity. Studies with 5-fluorouracil confirmed that dietary changes profoundly affect the outcome of cancer chemotherapy.

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INTRODUCTION

The general subject of this research project is the effect of diet and nutrition on the efficacy and toxicity of chemotherapy in women with breast cancer. More specifically, the research focuses on the interactions of a micro-nutrient, folic acid, with chemotherapeutic drugs frequently used clinically in women with breast cancer in either the adjuvant or metastatic setting. The hypothesis to be tested is whether dietary supplementation with a non-toxic nutrient, folic acid, may reduce the toxicity and increase the efficacy of chemotherapy in women with breast cancer. The purpose of the research is to better understand the effect of folate metabolism and varying dietary folate levels on the cellular pharmacology and clinical toxicity of chemotherapeutic agents and then utilize this knowledge to decrease the toxicity and increase the effectiveness of these drugs. The scope of the research involves *in vitro* studies with cell lines to assess folate-drug interactions and *in vivo* assessments in rats of folate-chemotherapeutic drug interactions.

BODY

Task 1. *In vitro* assessment of folate-drug interactions

- Determine the impact of folate levels on the cytotoxicity of 5-FU, doxorubicin and hydroperoxycyclophosphamide

The cell lines described in the grant application: MCF-7 (mammary adenocarcinoma); BT-474 (mammary ductal carcinoma); SK Br3 (mammary ductal carcinoma); MDA-MB-435 (mammary adenocarcinoma); and Hs578 Bst (human breast fibroblast cells) were located in tissue repositories and obtained. They were grown out in their original media and adapted to growth in media that can be formulated as folate-free. In the meantime, experiments were performed with TK6 human lymphoblastoid cells instead of the RPMI 1788 human lymphoblastoid cells described in the grant. We decided that the TK6 cells were preferable because we already had them adapted to growth in media that can be formulated folate-free, and a variant mutated at the p53 gene (WTK1) was available in our laboratory. The latter cells proved useful to investigate the role of p53 mutations on the interaction of folate metabolism and chemotherapeutic drugs.

As noted in last year's Annual Report, we encountered considerable difficulty identifying a source of hydroperoxycyclophosphamide. It is not commercially available, and most of the currently synthesized drug is committed to studies of bone marrow purging in preparation for bone marrow transplantation. We were able to obtain a small quantity from Dr. Carol Miller at the Johns Hopkins Medical School. Our plan was modified to perform the studies described in the grant with 5-FU, methotrexate and doxorubicin, and use ethyl methanesulfonate (EMS) as a surrogate for cyclophosphamide, since its mechanism of action is similar. We will then repeat critical experiments with hydroperoxycyclophosphamide when the experimental conditions have been defined.

The TK6 and WTK1 cells were cultured for 3 days in folate-replete or -free RPMI medium, then treated overnight with EMS. Cells then were grown in complete medium for 7-8

days to go through phenotypic lag. Cells were seeded for cloning efficiency and for mutant selection in the presence of 10 uM 6-thioguanine into 96 well microtiter plates. At 10-14 days, colony growth was scored and cloning efficiency calculated by use of the Poisson relationship. The mutant frequency is the ratio of the mean cloning efficiency in the presence and absence of 6-thioguanine.

After synthesis of cDNA, amplification was done in two rounds of nested PCR in a Perkin-Elmer 2400 Thermal Cycler. The final product was run on a 1% agarose gel, stained with ethidium bromide and observed under UV light. The product was excised, Gene Cleaned and sequenced with an ABI 373 sequencer. Some clones were studied further by multiplex PCR.

Treatment of the cell lines with increasing concentrations of EMS resulted in a progressive decline in cloning efficiency. Figure 1 shows the results of 5 separate experiments in which TK6 and WTK1 cells were incubated in folate-replete or deficient media and then treated with EMS in the concentration range of 0 to 50 ug/ml. WTK1 cells were more resistant to the cytotoxic effects of EMS than TK6 cells in both folate replete and deficient media. Folate

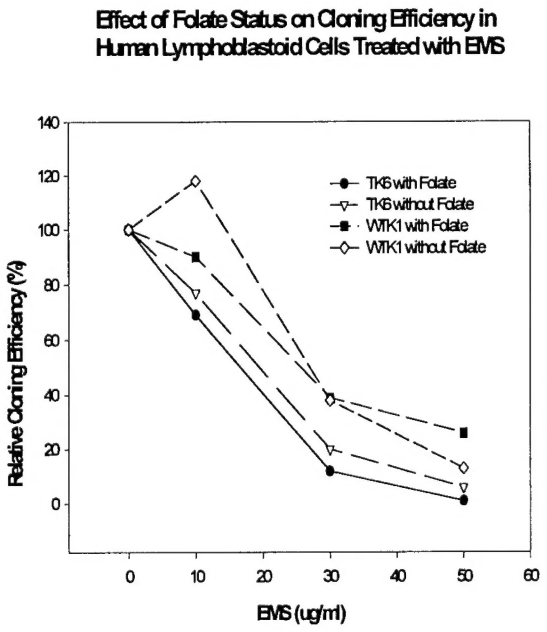


Figure 1

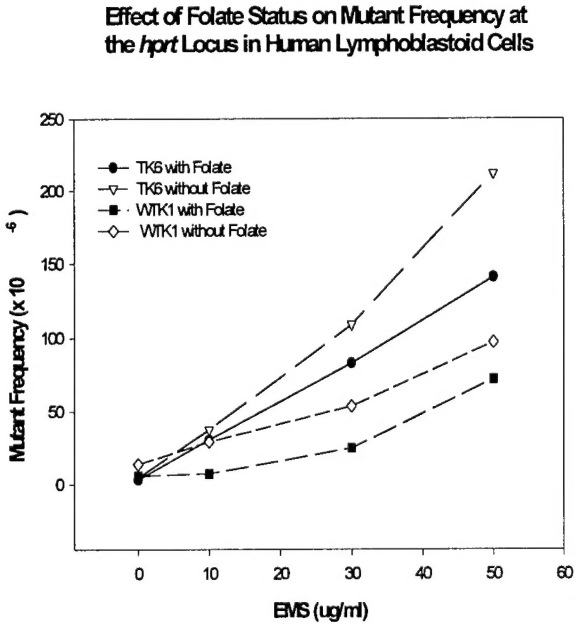


Figure 2

deficient TK6 were more resistant to the toxic effects of EMS than folate replete cells. However, there was considerable variability among the experiments, and statistical analyses indicated that the slopes for the cloning efficiencies were similar.

The mutant frequencies at the *HPRT* locus determined from these 5 experiments are shown in Figure 2. After treatment with EMS, the mutant frequencies were higher in TK6 than WTK1 cells in both folate replete and deficient medium. The mutant frequencies were higher in folate deficient than replete cells, but the effect was greater in TK6 cells. Statistical analysis of

the slopes indicated significantly different *HPRT* mutation dose-response relationships at the 0.01 level.

Molecular analyses of 152 6-thioguanine resistant mutant clones are shown in detail in Table 2 of the appended manuscript (the full table could not be included in this application because of its length). A summary of this data is presented here as Table I. The predominant mutation (63%) in both cell types grown in the presence or absence of folic acid was a G>A transition on the non-transcribed strand. These transitions were mainly at non-CpG sites, particularly when these bases were flanked 3' by a purine or on both sides by G:C base pairs. The section of exon 3 that contains six guanines in a row was especially susceptible to mutation (19 of the 95 G>A mutations). A smaller number of G>A transitions occurred on the transcribed strand, reflected as C>T transitions (14%) and were more common in the folate-deficient WTK1 cells. The most striking difference between the folate-replete and deficient cells was an increased frequency of deletions in the cells of both types grown under low-folate conditions (17%) as compared to replete cells (4%). In addition, four mutations in low folate cells resulted in no cDNA and no obvious change in the nine *HPRT* exons and could represent translocation events, or deletions that interfered with RNA splicing. Therefore the total deletion frequency could be as high as 17 of 75 (23.7%) in the low folate mutations.

The predominant mutation in EMS-treated folate-replete WTK1 cells was a G>A transition. EMS is mutagenic by reaction with the O^6 and N^7 positions of guanine. O^6-ethylguanine is mutagenic by pairing with thymine during replication, while N^7-alkylation products lead to apurinic sites that are processed by base excision repair and may cause mutations by defective repair or by mis-incorporation (1-5). Under folate-replete conditions, most mutations (69%) in the WTKI cells were G>A transitions, suggesting that the deficiency of the AGT repair mechanism was a major contributing factor to persistent mutations. The smaller number of genomic deletions (8%) probably reflects error-prone base excision repair, because base excision repair defective cell lines exhibit increased percentages of deletion mutations after EMS treatment (6,7). The mutational spectrum in the p53-competent TK6 cell line was similar to WTK1 cells after EMS treatment in folate-containing medium, showing 76% G>A transitions but no deletions. This observation suggests that the p53 gene product does not have a major influence on the molecular spectrum after treatment with monofunctional alkylating agents.

Folate deficient TK6 and WTK1 cells, like folate replete cells, showed a predominance of G>A transitions. However they also exhibited an increased percentage of deletions compared to folate-replete cells. This finding supports and confirms our previous report that folate-deficient CHO cells had more intragenic deletions after EMS treatment than folate replete cells (8). Folate deficient WTK1 cells were found to have a higher percentage of C>T transitions (21%) than either folate replete WTK1 cells (10%) or TK6 cells regardless of folate status (16% replete, 8% deficient). This higher percentage of C>T transitions may represent persistence of G>A transitions on the transcribed strand of these folate-deficient, p53 mutant cells. Alternatively, O^2-ethylcytidine may act as uracil and code for thymine (9) giving the pathway: O^2-ethylcytidine $\rightarrow$ U $\rightarrow$ T.

Table I
Summary of mutation types in the HPRT gene in 6-thioguainine resistant human lymphoblastoid cells exposed to EMS

Mutation Type (No. of mutations)	NTS	Folate Replete		Low Folate	
		TK6 (38)	WTK1 (39)	TK6 (37)	WTK1 (38)
Transitions					
GC>AT	G>A	29 (14)[a]	27 (12)[a]	21 (10)[a]	18 (7)[a]
	C>T	6	4	3	8
AT>GC	A>G	0	1	0	0
	T>C	0	1	0	0
Transversions					
GC>TA	G>T	0	2	2	1
	C>A	1	0	0	0
GC>CG	G>C	0	0	2 (1)[a]	0
	C>G	0	0	0	0
AT>TA	A>T	0	0	1 (1)[a]	1 (1)[a]
	T>A	0	0	0	0
AT>CG	A>C	0	0	0	0
	T>G	0	0	1	0
Genomic deletion		0	3	3	10
"new exon"		1			
no change		1[b]	1[c]	4[d]	

NTS = nontranscribed strand
[a]mutations which affected splicing
[b]exon 8 exclusion in cDNA with no change in genomic found
[c]exon 4 exclusion in cDNA with no change in genomic found
[d]four mutations with all nine exons present in genomic DNA and no change in the sequence of exons 1, 7, 8 and 9.

Calculations of the ID_{50} for the breast cancer cell lines by EMS are shown in Table II. The cells were cultured in either folate-deficient medium or in 2 concentrations of folate, 2 ug/ml (a concentration found in many standard culture media) and 50 ug/ml. The data in Table II suggest that higher folate levels tend to protect against the toxic effects of EMS. This observation is consistent with our original animal studies, in which rats on high folate diets treated with cyclophosphamide had less toxicity than folate deficient rats. However, this data does not support the observation that breast cancers in folate deficient rats were less sensitive to alkylating agent.

Table II. The Effect of Culture Medium Folate Level on the Concentration of EMS that Inhibits Growth by 50% of Breast Cancer Cell Lines of Varying Estrogen Receptor and Erb B2 status.

				Folate Concentration (ug/ml)		
Cell Line	**Pathology**	**ER**	**Erb B2**	**0**	**2**	**50**
MCF 7	Adenocarcioma	+	+/-	0.56*	0.56	0.70
BT-474	Ductal carcinoma	+	++	0.38	0.44	0.40
SK Br 3	Ductal carcinoma	-	+++	0.21	0.19	0.22
MDA-MB-435	Adenocarcinoma	-	-	0.07	0.08	0.11
MADB 106	Rat adenocarcinoma			0.43	0.50	0.46
TK 6	Lymphoblast			0.013	0.013	

* ID_{50} in mg/ml EMS

During the coming year the ID_{50} 's for doxorubicin and 5-fluorouracil will be measured in the same breast cancer cell lines as outlined in the original Statement of Work.

- Determine mechanism of folate-drug interactions
  - Assess DNA synthesis
  - Perform alklaline/neutral elutions to determine DNA lesions
  - Western blots to determine topoisomerase II/PGP exression where appropriate (budget support not approved)
  - Enzymatic/drug uptake studies for protein function

While the pharmacologic mechanism that accounts for the amelioration of alkylating agent-induced toxicity by folate supplementation illustrated by the data in Table II requires further elucidation, our laboratory reported evidence last year in our Annual Report that altered glutathione levels may be involved. It is well established that glutathione protects against the cytotoxicity of alkylating agents (reviewed in 10). Since folate metabolism is involved in the synthesis of the three amino acids that constitute glutathione (glutamate, cysteine and glycine), we measured glutathione levels in liver samples from rats on diets of varying folate content and treated with increasing doses of cyclophosphamide. As shown in Figure 3, below, glutathione levels increased in the order: folate deficient<folate replete<high folate, and this result was statistically significant ($p<0.0001$) using analysis of variance. Moreover, glutathione levels were directly proportional to cyclophosphamide dose ($p<0.0001$), and there was a significant dose-diet interaction ($p = 0.012$). These results suggest that the combination of folate supplementation and cyclophosphamide promotes higher levels of glutathione production and protection against toxicity.

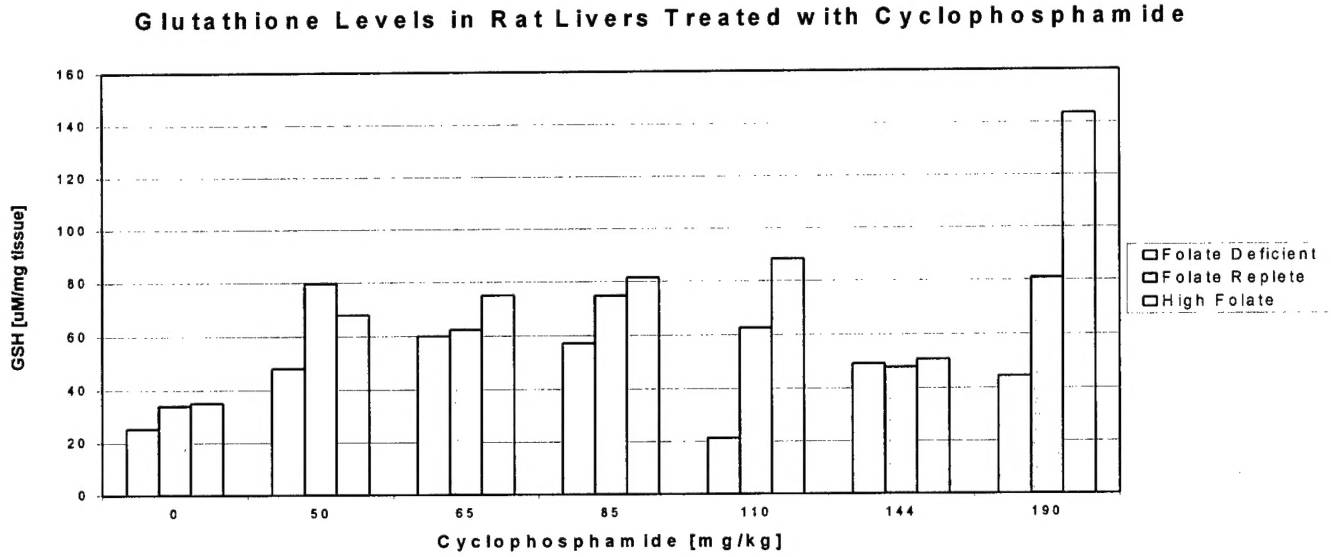


Figure 3.

During the coming year other mechanistic studies will be performed as appropriate, as outlined in the original Statement of Work.

Task 2. *In vivo* assessment of folate drug interactions

- Determine the effect of folate status on tumor growth rate
- Determine the effect of folate status on drug efficacy
- Determine the effect of folate status on drug toxicity

These experiments are proceeding as described in the grant application. We elected to start with the toxicity studies, since these experiments are the most time-consuming because of the numerous assays for toxicity. In addition, we plan to use the results for dose-finding for the efficacy studies.

Weaning female Fischer 344 rats were maintained on either standard rat chow (Teklad 7012) or a Purified Diet containing either no folic acid or 2 mg folic acid/kg of diet, as previously reported by our laboratory (11). Some rats on the folate-containing diet received additional folic acid, 50 mg/kg, intraperitoneally (IP) daily. After 5 weeks on these diets, the rats were injected with a single dose of either cyclophosphamide or 5 fluorouracil (5-FU) IP. Blood was obtained for laboratory determinations prior to the chemotherapy injection, and on days 4, 9 and 14 afterward. Surviving rats were sacrificed on day 14 and the livers collected and frozen.

As reported in last year's Annual Report, measurements of hepatic folate levels by the *Lactobacillus casei* method (11) gave the following results: standard rat chow, 34.0 μg/g; folate replete diet, 32.1 μg/g; folate deficient diet, 9.1 μg/g; high folate animals, 45.8μg/g. These results indicate that the dietary conditions caused important differences in tissue folate levels.

The number of deaths in each dietary group (6 animals/group) after treatment with increasing doses of cyclophosphamide, and the measurements of growth, bone marrow toxicity (HCT, WBC), renal toxicity (BUN), liver toxicity (LDH, SGPT) and cardiac toxicity (CPK) were presented in last year's Annual Report. During the past year these data were analyzed statistically.

The following conclusions can be drawn from this experiment:

1. The median lethal dose (LD_{50}) are:

cereal control	232 mg/kg
folate deficient	154 mg/kg
folate replete	159 mg/kg
high folate	148 mg/kg

- The cereal control was significantly different from the other groups; there was no significant difference among the folate groups.

2. There were no significant differences in rat weights at 6 weeks on the various diets.

3. Rat weight at 6 weeks did not predict survival after cyclophosphamide (ctx) treatment.

- Diet group and ctx dose level influence survival.

4. Weight gain (growth) during the first 6 weeks was the same in the various dietary groups.

5. Weight at week 7 did not differ among the dietary groups at any of the ctx dose levels.

6. At week 8, the cereal controls were significantly heavier than the other groups at the 85 mg/kg dose, while the folate deficient rats lost significantly more weight than the other groups at the 110 mg/kg dose.

7. At week 6 (pre-treatment) the high folate group had a significantly higher BUN than the other dietary groups.

8. There was no difference in WBC or HCT pre-treatment among the dietary groups.

9. The cereal control group generally had higher HCT's during the treatment than the other groups.

10. On day 9, the WBC was higher in the high folate group at 50 mg/kg, and higher in the cereal group at 65 mg/kg.

11. The BUN generally was higher in the high folate group on days 9 and 14 than in the other dietary groups.

12. After logistic regression, diet and dose caused the main effects; pre-treatment BUN, WBC and HCT were not significant when added to diet and dose, or just dose.

13. After logistic regression, deaths were predicted by dose, diet, WBC and BUN on day 4.

14. Measurements of covariance indicated that:

- With regard to HCT, there were interactions of diet and time and dose and time, indicating that different diets and different doses act differently over time. Therefore all of the curves are different over time from each other.
- With regard to WBC, dose and diet have a differential effect over time; there is no diet interaction with time. Therefore dose rather than diet is more important for interaction over time for WBC. Diet has a more unique effect on dose over time on HCT than WBC.
- With regard to BUN, there is a dose-time but not diet-time interaction, but diet has an effect on BUN.

The above results suggested that the causes of death were bone marrow suppression, an expected effect of cyclophosphamide treatment, and renal failure, and unanticipated effect. The renal failure appeared to be most prominent in the high folate dietary group. Therefore histologic examination of kidneys from rats in the different dietary groups was performed by a pathologist at the University of Vermont. He found that the kidneys from rats on the 4 diets alone were normal. Similarly, the kidneys from rats on the cereal control diet and treated with chemotherapy also were normal. However, kidneys from rats on the low folate and folate replete diets showed focal tubular regeneration, while the kidneys from rats on the high folate diet showed acute tubular necrosis, often marked. These results indicate that the combination of a purified diet and chemotherapy is nephrotoxic compared to a cereal-based diet and chemotherapy. The addition of high levels of folic acid to the purified diet and chemotherapy exacerbates the renal toxicity. There have been previous suggestions in the literature that high levels of folic acid may cause kidney damage. For example, Achon and colleagues recently reported that high dietary folate supplementation of rats was associated with impaired dietary protein metabolic utilization and higher urinary nitrogen elimination (12). Fetuses of folate supplemented dams had lower body weight and shorter vertex-coccyx length compared to unsupplemented dams (12). Klingler et al found that folic acid administered to rats in doses of 100, 200, 300 and 400 mg/kg of body weight resulted in changes in tubular morphologic features and renal function, and the severity of the changes were proportional to dose (13). Similarly, Schubert and colleagues reported that repeated injections of folic acid, 250 mg/kg, to rats caused severe chronic kidney damage (14). Renal toxicity in our animals occurred at much lower folate levels (50 mg/kg), suggesting a synergistic interaction between diet, chemotherapy and folic acid. To the extent that these studies are relevant to humans, they suggest that there may be an optimal dose of folic acid supplementation to modify the effects of cancer chemotherapy.

Statistical analyses confirmed the resistance to alkylating agent toxicity shown by the animals on the Teklad 7012 rat chow. This resistance is not mediated by dietary folate levels or hepatic glutathione levels. Other possible mechanisms we are exploring to explain the reduced toxicity include increased quenching of reactive oxygen species or the enhanced induction of the cytochromes P-450 drug/toxin metabolizing system and mixed function oxidases in animals fed cereal-based diets such as Tekad 7012 (15).

Toxicity studies are underway with 5 FU. Table III shows the results to date. These studies suffered a major set-back because 3 groups of animals (an untreated control group, 190 mg/kg and 250 mg/kg) were infected with a virus, sialodacryoadenitis, during the experiment. Therefore these animals are not evaluable. This loss caused a 3 month delay in the progress of these experiments and considerable expense for replacement animals and technician time.

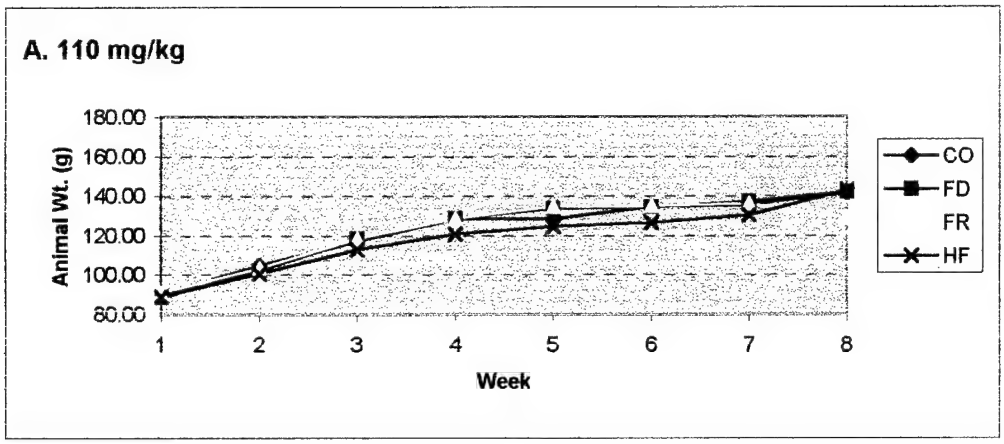
Table III. Effect of diet on 5-FU toxicity in rats with death as an end-point.

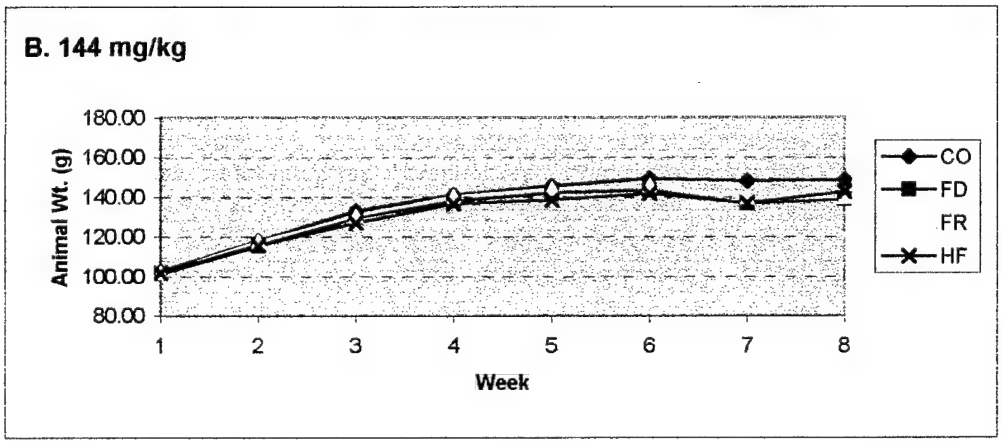
	Diet			
Dose of 5-FU (mg/kg)	**Control**	**Folate Deficient**	**Folate Replete**	**High Folate**
110	0	0	1	4
144	1	1	1	3
190	0	0	0	0
250	3	0	2	1
325	2	0	1	0
420	1	0	1	5

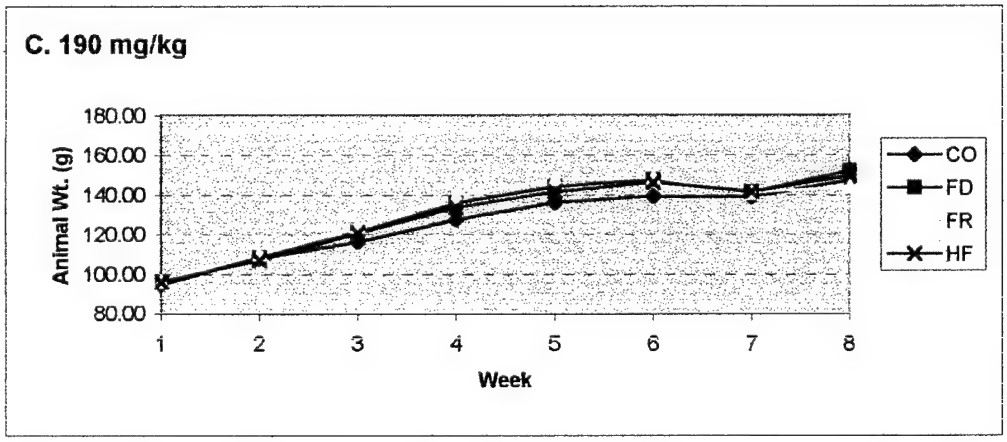
The weight changes in the rats on the various diets are shown in Figure 4. During the first 5 weeks growth rates were similar, indicating that the diets are nutritionally adequate to support growth. In weeks 7 and 8, rats that were injected with 5-FU showed a reduction in growth rate and in some cases weight loss. However, there was no clear dietary effect.

The results of analyses of bone marrow, liver, kidney and cardiac toxicity at the various 5-FU dose levels are shown in Figures 5-10. In these figures, the diets are: CO, control rat chow (cereal-based); FD, folate deficient; FR, folate replete; HF, high folate. Hematocrit, white blood cell counts, and BUN were measured prior to therapy and 4, 9 and 14 days after chemotherapy. Measurements of LDH, SGPT and CPK were done only on day 14 because larger volumes of blood were required and these volumes could only be obtained when the animals were exsanguinated. For the white blood count, the Coulter Counter (Model ZBI) was used. Analyses of BUN, LDH, SGPT and CPK were performed using Sigma Diagnostics (St. Louis, MO) Procedures No. 66-UV, 500, 505 and 661, respectively. As was the case with cyclophosphamide, the combination of 5-FU and a high folate diet was associated with more evidence of renal failure.

Figure 4. Weight Changes in Rats on Diets of Differing Folate Content Treated in Week 6 with 5-FU.

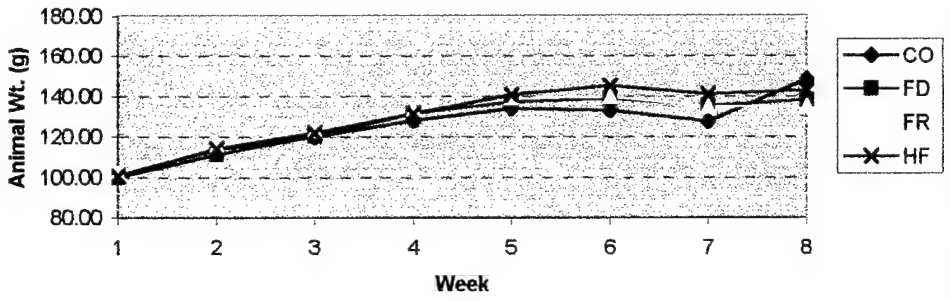




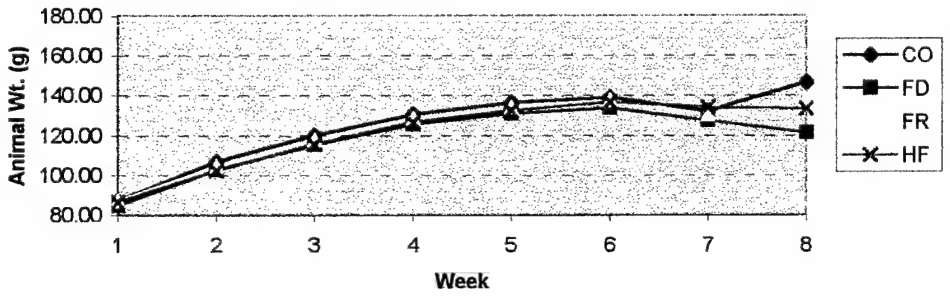


CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

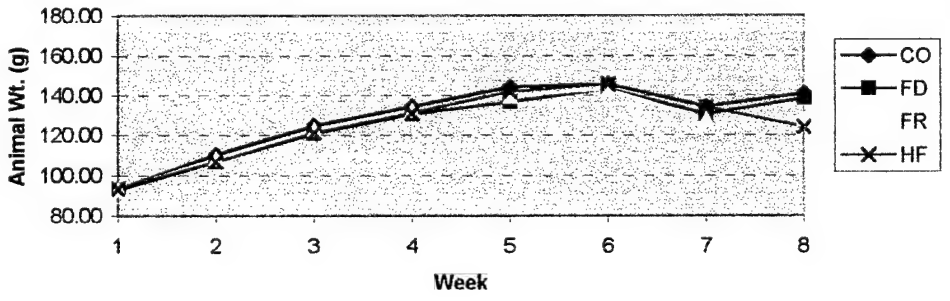
D. 250 mg/kg



E. 325 mg/kg

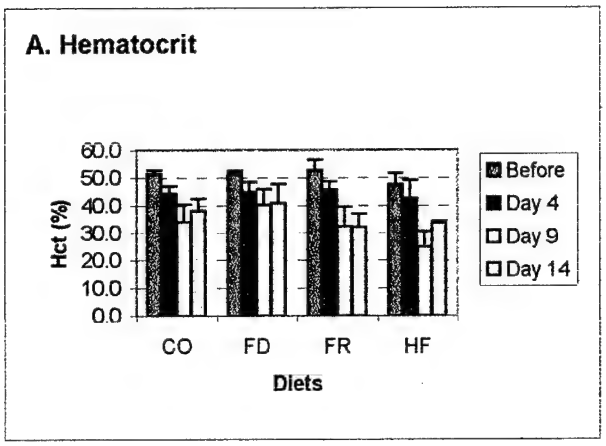


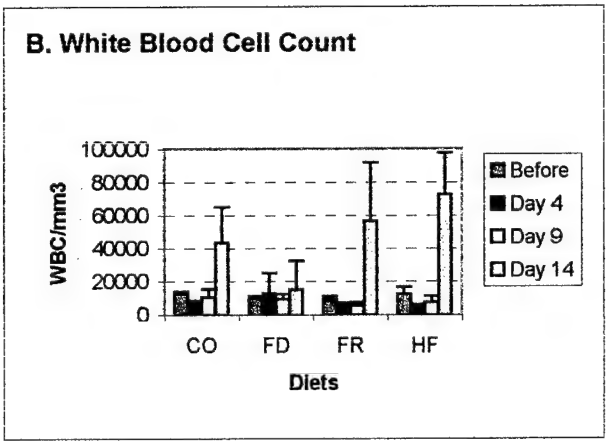
F. 420 mg/kg

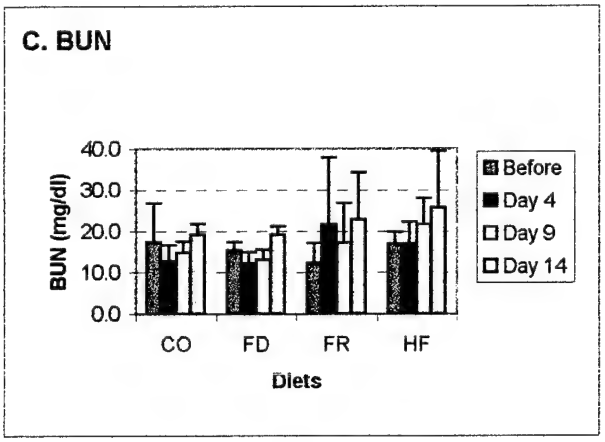


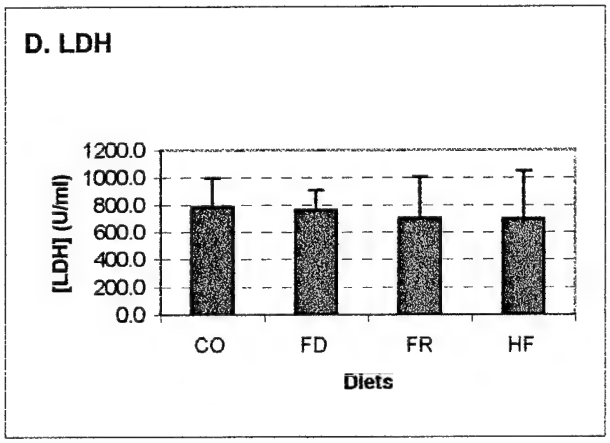
CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

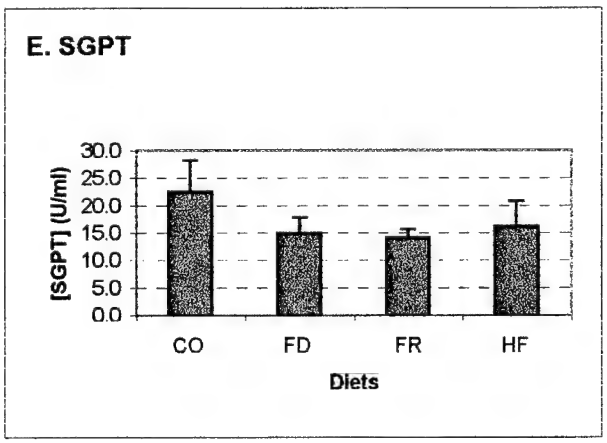
Figure 5. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with 5-FU, 110 mg/kg.

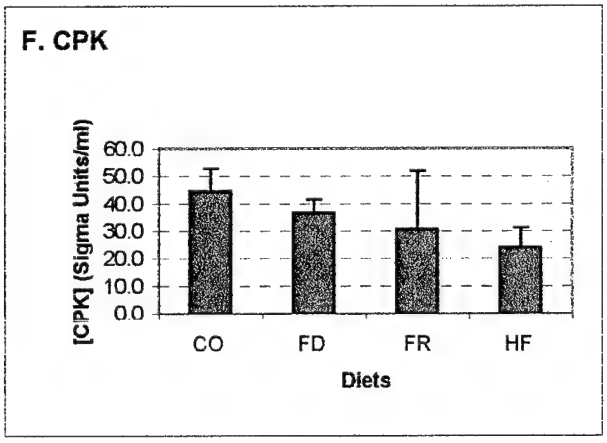






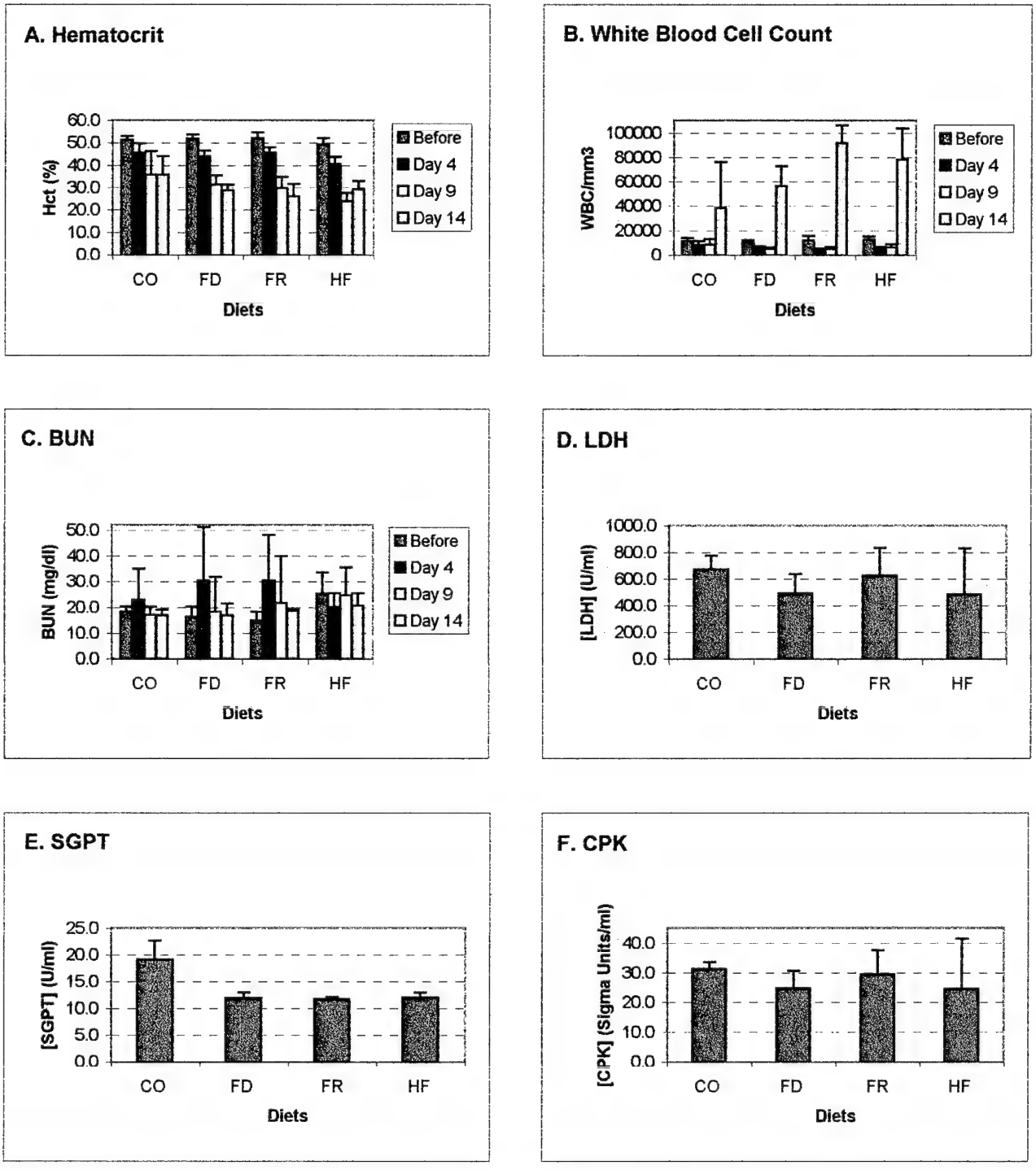






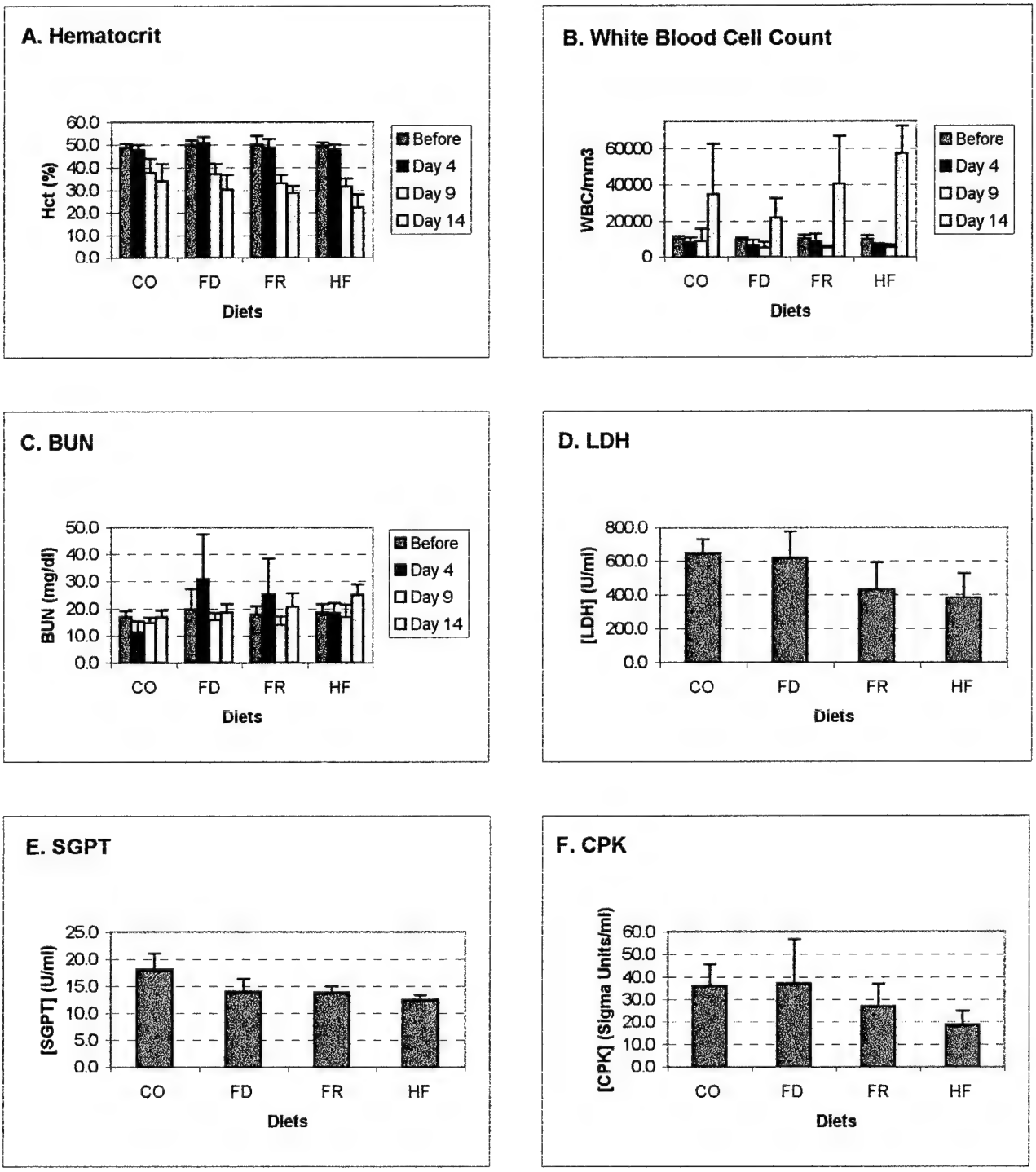
CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

Figure 6. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with 5-FU, 144 mg/kg.



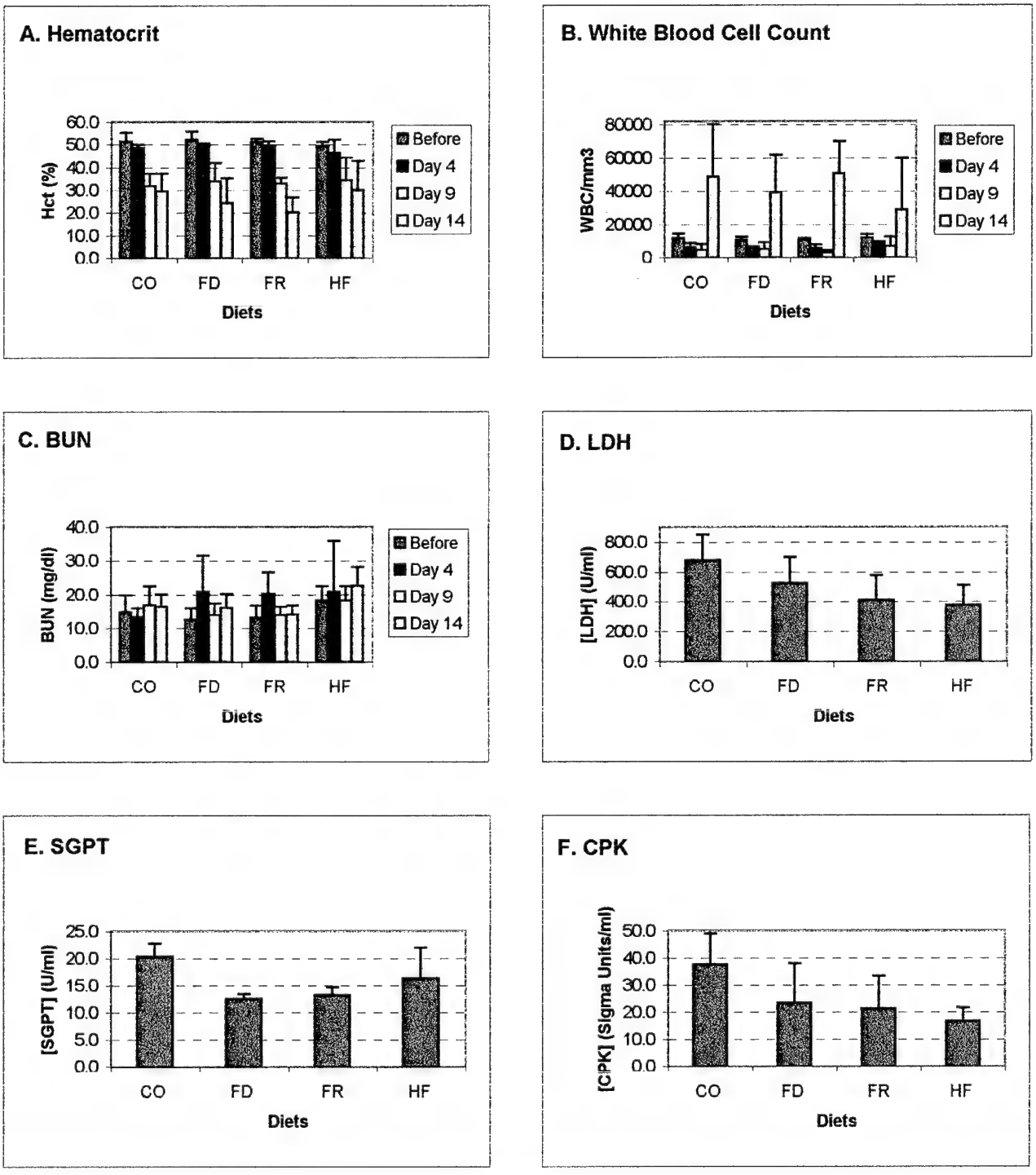
CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

Figure 7. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with 5-FU, 190 mg/kg.



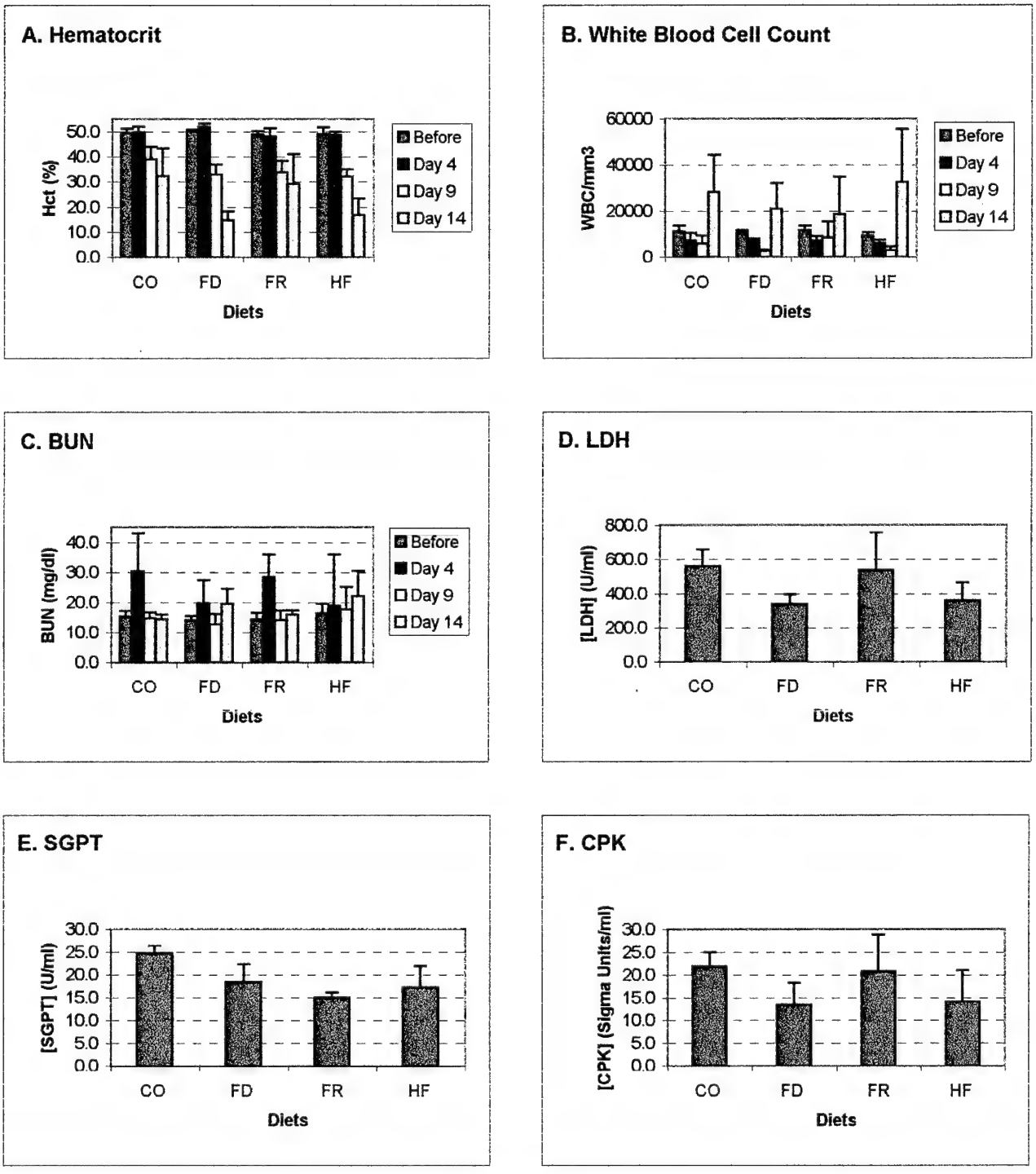
CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

Figure 8. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with 5-FU, 250 mg/kg.



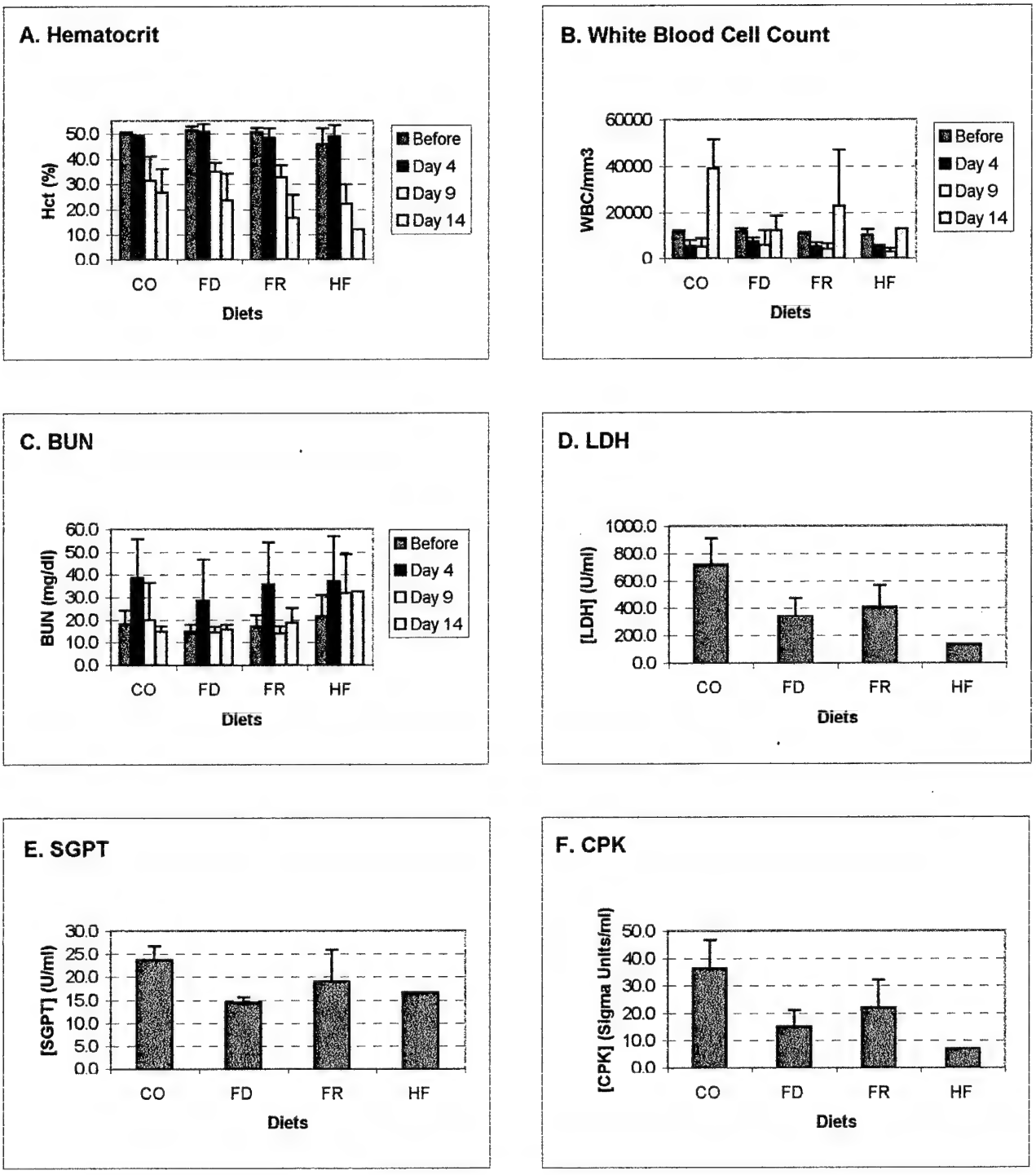
CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

Figure 9. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with 5-FU, 325 mg/kg.



CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

Figure 10. Bone Marrow, Liver, Kidney and Cardiac Toxicity in Rats Measured Before and 4, 9 and 14 Days after Treatment with 5-FU, 420 mg/kg.



CO = Cereal based diet, FD = Folate deficient diet, FR = Folate replete diet, HF = High folate diet

KEY RESEARCH ACCOMPLISHMENTS

- Studies of the interaction of folate metabolism and chemotherapeutic drugs at the molecular level in human lymphoblastoid cell lines supported our model to explain the synergy between nutritional folate deficiency and alkylating agents.

- A previously unrecognized relationship between folate metabolism and cellular glutathione levels was confirmed in a larger number of rat liver samples.

- The inhibitory concentrations (ID_{50}) of alkylating agent for 5 breast carcinoma cell lines cultured in media of varying folate levels were identified.

- Statistical analyses of toxicity studies with cyclophosphamide in rats indicated that:
  - Rats on a cereal-based diet were significantly more resistant to the toxicity of cyclophosphamide than rats on a Purified Diet.
  - There was no significant difference among rats on the Purified Diet.
  - Diet and dose caused the main effects; pre-treatment BUN, WBC and HCT were not significant when added to diet and dose, or just dose.
  - Deaths were predicted by dose, diet, WBC and BUN on day 4.

- The combination of a purified diet and chemotherapy was nephrotoxic compared to a cereal-based diet and chemotherapy. The addition of high levels of folic acid to the purified diet and chemotherapy exacerbated the renal toxicity. Therefore there may be an optimal dose of folic acid supplementation to modify the effects of cancer chemotherapy.

REPORTABLE OUTCOMES

Manuscript: Branda, R.F., O'Neill, J.P., Brooks, E.M., Trombley, L.M., Nicklas, J.A. The effect of folate deficiency on the cytotoxic and mutagenic responses to ethyl methanesulfonate in human lymphoblastoid cell lines that differ in p53 status. Submitted.

Abstract and Presentation: Branda, R.F., O'Neill, J.P., Brooks, E.M., Trombley, L.M., Nicklas, J.A. P53 activity modulates the effect of folate deficiency on genetic damage caused by alkylating agents in human lymphoblastoid cells. Proc. AACR 41:69, 2000. Presented at the Annual Meeting of the AACR, April, 2000

Funding applied for based on work supported by this award:

- Effect of Folate on Chromosomes in Breast Cancer, NCI, R.F. Branda, P.I.
- Effects of St. John's Wort and Vitamin E on Breast Cancer Chemotherapeutic Agents, DOD, R.F. Branda, P.I.

CONCLUSIONS

The experiments described in this Annual Report analyze the relationship between a micro-nutrient, folic acid, and the toxicity of chemotherapeutic drugs used to treat women with

breast cancer. Our studies of the interaction of folate metabolism and alkylating agents at a molecular level confirm that intragenic deletions and G>A transitions are the predominant chromosomal changes, and indicate that human cells manifest the same mutational spectra as rodent cells. Since the findings reported here occurred in human cell lines, they may be clinically relevant. Our results suggest that cells expressing p53 activity exhibit a higher rate of mutation induction but are more sensitive to the toxic effects of alkylating agents than those lacking p53 activity. Folate deficiency tends to reduce toxicity but increase mutation induction after alkylating agent treatment. TK6 cells are deficient in alkyltransferase activity and resemble in this regard bone marrow myeloid precursors (16). The bone marrow is a frequent target organ for alkylating agent-induced transformation, and acute myelogenous leukemia has been reported following exposure to cyclophosphamide, melphalan and busulfan as single agents and to the combination of nitrogen mustard and procarbazine (17). Cytogenetic analyses indicate that transformation to myelodysplasia and acute leukemia is associated with deletions and translocations (17). Thus the combination of alkylating agent exposure and nutritional folate deficiency in a cell type deficient in alkyltransferase but expressing wild-type p53 creates an environment that increases the risk of developing carcinogenic genetic changes. The addition of a mutation at the p53 locus would alter further the biology of a developing tumor cell, in that loss of p53 activity reduces toxicity from monofunctional alkylating agents, and those cells lacking p53 activity and folate are most resistant. The corollary of these observations is that correction of nutritional folate deficiency may reduce the risk of developing carcinogenic genetic changes in normal cells after alkylating agent exposure and enhance the sensitivity to alkylating agents in p53-mutant malignant cells. The latter interpretation may explain, at least in part, our observation that mammary carcinoma was more resistant to cyclophosphamide chemotherapy in folate deficient rats than in folate replete animals (11). Our studies at a cellular level in rat livers from animals maintained on diets of varying folate content and treated with cyclophophamide confirm that folate metabolism can modulate GSH levels. Therefore, dietary supplementation with folic acid may represent a new and non-toxic approach to change the levels of an important determinant of alkylating agent toxicity. Finally, our animal studies indicate that there is an optimal amount of dietary folate to modify the toxicity of alkylating agents. Moreover, very high doses of folate added to chemotherapy can lead to kidney damage. Therefore future protocols in patients will need to carefully consider the amount of supplemental folic acid administered. In addition, it may be necessary to alert patients receiving chemotherapy who are taking dietary supplements that high doses of folic acid should be avoided.

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APPENDICES

Award Number: DAMD17-98-1-8345

TITLE: Effect of Folate on the Efficacy and Toxicity of Cancer Chemotherapy

PRINCIPAL INVESTIGATOR: Richard F. Branda, MD

Appendice 1 – Poster, Proceedings of the American Association For Cancer Research, Volume 41, March 2000

Appendice 2 – Manuscript, The Effect Of Folate Deficiency On The Cytotoxic And Mutagenic Responses To Ethyl Methanesulfonate In Human Lymphoblastoid Cell Lines That Differ In p53 Status

amplification for either *PIK3CA* or *PIK3CB*. In contrast, none of the 61 adenocarcinomas showed a copy number ratio greater than 2 neither for *PIK3CA* nor for *PIK3CB*. Only 4/62 tumors had a ratio greater than 2. This data suggests that the amplification of genes in the PI3-Kinase pathway may be involved in the development of squamous cell carcinoma but not adenocarcinoma of the lung. Further investigations on PI3-Kinase pathway genes are under way to determine the functional significance of copy number increase of these genes in lung cancer development and progression. (Supported in part by a Parker B. Francis Fellowship).

#440 SPECIFIC CHROMOSOMAL CHANGES IN MOUSE LUNG ADENOCARCINOMA CELL LINES DETECTED BY SPECTRAL KARYOTYPING. Linda M Sargent, J R Senft, D T Lowry, A M Jefferson, F Tyson, and S H Reynolds, *National Institute for Occupational Safety and Health, Morgantown, WV, and National Institute of Environmental Health Sci, Research Triangle Park, NC*

Adenocarcinoma is rapidly becoming the most common form of lung cancer in the United States. The difficulty in obtaining lung cancer families and the problems in obtaining samples for various stages of human lung adenocarcinoma have lead to the use of primary lung tumors in mice as an experimentally manipulable model for human lung adenocarcinoma. Chromosomal changes in mouse lung tumors have been reported using standard chromosome banding analysis. Due to the difficulty in identification of marker chromosomes based on banding alone, we have analyzed 12 early passage mouse lung adenocarinoma cell lines by Spectral Karyotyping (Applied Spectral Imaging, Carlsbad California). An entire copy of chromosomes 6 (78%) and 15 (78%) were gained while chromosome 7 (67%) and 14 (33%) were lost. There was a gain of a portion of chromosomes 1 (67%) and 2 (56%) and a loss of a portion of chromosomes 4 (67%) and 8 (44%). The minimal region of alteration is 1G1,2F2, 4C6 and 8B3. The minimal regions of alteration of chromosomes 1, 2, 4 and 8 contains putative susceptibility genes for mouse lung adenocarinoma. In addition, the deleted regions contain linkage groups that are altered in human adenocarcinoma. Due to the conservation of linkage groups between mouse and human, the identification of susceptibility genes in the mouse may aid in the identification of similar genes in the human population.

#441 RELATIONSHIPS BETWEEN DNA INCORPORATION, MUTANT FREQUENCY, AND LOSS OF HETEROZYGOSITY AT THE *TK* LOCUS IN HUMAN LYMPHOBLASTOID CELLS EXPOSED TO AZT. Quanxin Meng, T. Su, O. A Olivero, M. C Poirier, X. Shi, X. Ding, and V. E Walker, *National Cancer Inst, Bethesda, MD, and Wadsworth Ctr, NYSDOH, Albany, NY*

The purpose of this study was to investigate the potential mechanisms of AZT mutagenicity and carcinogenicity by determining the AZT-DNA incorporation, AZT-induced thymidine kinase (*TK*) mutant frequencies (M*f*s), and the percentage of loss of heterozygosity (LOH) in AZT-induced *TK* mutants in the human lymphoblastoid cells. Cells were exposed to 300 μM AZT for 0, 1, 3, or 6 days, or to 0, 33, 100, 300, or 900 μM AZT for 3 days (n = 5 flasks/group). An AZT-RIA and a cell cloning assay were used to measure AZT-DNA incorporation and *TK* M*f*s, respectively. AZT was incorporated into DNA in a dose-related manner at concentrations up to 300 μM, above which no further increase was observed. *TK* M*f* increased with the extended duration and with incremental concentrations of AZT exposure. There was a positive correlation (P = 0.036, coefficient = 0.903) between AZT-DNA incorporation and AZT-induced *TK* M*f*s, suggesting that AZT incorporation into cellular DNA has a direct role in the genotoxicity of AZT. Southern blot analyses indicated that 84 % of AZT-induced mutants were attributable to LOH, consistent with the known mechanism of AZT as a DNA chain terminator.

#442 ARSENITE INDUCED P53 ACCUMULATION THROUGH THE ATM-DEPENDENT PATHWAY. Ling-Huei Yih, and Te-Chang Lee, *Institute of Biomed Sci, Acad Sinica, Taipei, Taiwan ROC*

Accumulated evidence has shown that arsenite-induced cytogenetic alterations are associated with its carcinogenicity. In the present study, arsenite-induced DNA strand breaks were demonstrated by alkaline single cell gel electrophoresis (Comet assay) in human fibroblasts (HFW). As accompanied to the appearance of DNA strand breaks in arsenite-treated HFW cells, we observed p53 accumulation by immunoblotting and immunofluorescence techniques. In addition, p53 down-stream proteins, such as p21 and MDM-2, were significantly induced by arsenite treatment. Furthermore, the kinetic of arsenite-induced p53 accumulation was similar to that induced by X-ray irradiation, but different from that induced by UV irradiation. Wortmannin, an inhibitor of ATM-kinase and/or PI-3 kinase, inhibited arsenite- or X-ray irradiation-induced p53 accumulations, but did not alter UV irradiation-induced p53 accumulation. These results suggest the involvement of ATM in arsenite-induced p53 accumulation. To confirm this point, we also demonstrated that arsenite treatment, similar to X-ray irradiation, did not induce p53 accumulation in ATM defective cells, GM3395. In contrast, UV irradiation caused p53 accumulation in GM3395 cells. Therefore, our present study suggested that arsenite induced DNA strand breaks which may lead to p53 accumulation through an ATM-dependent pathway in HFW cells.

#443 FRAGILE SITE (FRA) UPREGULATION DEFINES A DISTINCT PATHWAY OF HEAD AND NECK (HN) TUMORIGENESIS. Julie G Izzo, Maranke I Koster, Vali A Papadimitrakopoulou, Adel K El-Naggar, Waun K Hong, and Walter N Hittelman, *U T M D Anderson Cancer Ctr, Houston, TX*

FRA expression is involved in chromosomal rearrangements (deletions, amplifications, exchanges) and is inducible by environmental factors and carcinogens. Previously we demonstrated that FRA expression at the 11q13 region is detectable *in vivo* as split signals of a region-specific probe during HN tumorigenesis and that it precedes gene amplification. To study the relationship between FRA expression and specific events, 15 HN tumor specimens (8 with, 7 without 11q13 amplification) with adjacent premalignant lesions (PL) and normal mucosa (AN) were examined for loss of heterozygosity (LOH) at 9p21 and 3p14 (loci of known FRA), 11q13 FRA upregulation and cyclin D1 (CCND1) expression. FRA expression was higher in PL adjacent to amplified tumors (mean±SD 4.7%±. 4 *vs* .78±. 03, p=. 0006), in these epithelia FRA frequency increased sharply (3 fold) in regions with CCND1 dysregulation. LOH at 9p21 was observed in 7/8 (88%) PL of amplified cases, always with concomitant dysregulated CCND1, while in only 1/7 (14%) PL of non amplified cases. In contrast 3p14 LOH was mainly found in PL of non amplified cases (6/7 [86%] *vs* /8 [25%]) without CCND1 dysregulation. Remarkably, ANL of amplified cases (without CCND1 or 9p21 abnormalities) harbored higher FRA expression than ANL of non amplified cases (mean±SD: 3.6%±. 3 *vs*.3±.0, p=. 0002), suggesting an inherent defect. Although no difference in tobacco exposure was evident between the two groups, the FRA expressing group demonstrated clinical evidence of field cancerization [multiple asynchronous primaries (3/8)/premalignant lesions (3/8)]. These data suggest the possible existence of genetically predetermined and distinct pathways to tumor development in the form of FRA with tobacco acting as a promoter. (NIH CA-52051)

#444 P53 ACTIVITY MODULATES THE EFFECT OF FOLATE DEFICIENCY ON GENETIC DAMAGE CAUSED BY ALKYLATING AGENTS IN HUMAN LYMPHOBLASTOID CELLS. Richard F Branda, J Patrick O'Neill, Elise M Brooks, Lucy M Trombley, and Janice A Nicklas, *Univ of Vermont, Burlington, VT*

Relatively little is known about the effects of diet on the toxicity of chemotherapy. Our laboratory previously has shown that deficiency of folic acid acts synergistically with alkylating agents to increase genetic damage at the *hprt* locus in Chinese hamster ovary cells *in vitro* and in rat splenocytes *in vivo*. The present studies extend these observations to human cells and investigate the role of p53 activity. The human lymphoblastoid cell lines TK6 and WTK1 are derived from the same parental cell line (WI-L2), but WTK1 expresses mutant p53. Treatment of folate replete or deficient cells with increasing concentrations (0-50 μg/ml) of ethyl methanesulfonate (EMS) resulted in significantly different *hprt* mutation dose-response relationships ($P<0.01$), indicating that folate deficiency increased the EMS-induced mutant frequency in both cell lines, but with a greater effect in TK6 cells. Molecular analyses of 153 mutant clones showed more intragenic deletions, complex chromosomal changes and G>T transitions in folate deficient as compared to replete TK6 cells, and a striking increase of C>T transitions in folate deficient WTK1 cells as compared to the other 3 groups. These results indicate that folate deficiency augments genetic damage in human cells, particularly those that express p53, and changes the mutational spectra caused by alkylating agents.

#445 EXPRESSION OF BASE EXCISION REPAIR ENZYMES IN RAT AND MOUSE LIVER IS INDUCED BY PEROXISOME PROLIFERATORS AND IS DEPENDENT UPON CARCINOGENIC POTENCY. Ivan Rusyn, Ronald G Thurman, Michael L Cunningham, and James A Swenberg, *NIEHS, RTP, NC, and Univ of North Carolina at Chapel Hill, Chapel Hill, NC*

Sustained elevation of cell replication and inhibition of apoptosis are considered main mechanisms of tumor promotion for peroxisome proliferators (PPs). A potential role of oxidative stress and DNA damage has also been proposed. In view of the possible formation of DNA adducts by PPs, DNA repair may be an important factor to consider in the mechanism of PPs. Here, the ability of PPs to induce expression of base excision repair enzymes was examined in livers of rats or mice fed PP-containing diets using an RNAse protection assay and quantitative RT-PCR. In mice, WY-14,643 (WY, 500 ppm, 1 wk), a potent carcinogen, caused a 3-fold increase in mRNA for 8-OH-dG glycosylase (OGG1), thymine DNA glycosylase (TDG), AP endonuclease (APE), N-methylpurine DNA glycosylase (MPG) and endonuclease III (Nth1). In contrast, administration of diisononyl phthalate (8000 ppm), a less potent carcinogen, had little effect. In rats, when WY (1000 ppm) and diethylhexyl phthalate (12000 ppm), a weak carcinogen, were given for 7 days, induction of OGG1, APE, and Nth1 was observed only in WY-treated animals. Since these two compounds cause a similar initial increase in cell proliferation, this effect could not be attributed solely to a rapid growth of liver mass. Moreover, WY (0-500 ppm; 34 and 90 d) induced both time- and dose-dependent increases in expression of OGG1, APE, Nth1 and MPG. Similar effects were observed with gemfibrosil (0-16000 ppm, 90 d), a rodent carcinogen, but not with dibutyl phthalate (0-10000 ppm, 34 and 90 d). Collectively, these data suggest that DNA base excision repair may be an important factor in PP-induced carcinogenesis and could provide further evidence supporting a role of oxidative DNA damage by PPs. (ES 9785)

THE EFFECT OF FOLATE DEFICIENCY ON THE CYTOTOXIC AND MUTAGENIC RESPONSES TO ETHYL METHANESULFONATE IN HUMAN LYMPHOBLASTOID CELL LINES THAT DIFFER IN p53 STATUS

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Keywords: Folate; alkylating agent; *HPRT* mutation spectrum; human lymphoblast cells; p53

ABSTRACT

Folic acid deficiency acts synergistically with alkylating agents to increase genetic damage at the *hprt* locus in Chinese hamster ovary cells *in vitro* and in rat splenocytes *in vivo*. The present studies extend these observations to human cells and, in addition, investigate the role of p53 activity on mutation induction. The human lymphoblastoid cell lines TK6 and WTK1 are derived from the same parental cell line (WI-L2), but WTK1 expresses mutant p53. Treatment of folate replete or deficient WTK1 and TK6 cells with increasing concentrations (0-50 μg/ml) of ethyl methanesulfonate (EMS) resulted in significantly different *HPRT* mutation dose-response relationships ($P<0.01$), indicating that folate deficiency increased the EMS-induced mutant frequency in both cell lines, but with a greater effect in TK6 cells. Molecular analyses of 152 mutations showed that the predominant mutation (63%) in both cell types grown in the presence or absence of folic acid was a G>A transition on the non-transcribed strand. These transitions were mainly at non-CpG sites, particularly when these bases were flanked 3' by a purine or on both sides by G:C base pairs. A smaller number of G>A transitions occurred on the transcribed strand (14%), and these were more common in folate-deficient WTK1 cells. There were more genomic deletions in folate deficient (17%) as compared to replete cells (4%) of both cell types. Mutations that altered RNA splicing were common in both cell types and under both folate conditions, representing 31% of the total mutations. These studies indicate that cells expressing p53 activity exhibit a higher rate of mutation induction but are more sensitive to the toxic effects of alkylating agents than those lacking p53 activity. Folate deficiency tends to reduce toxicity but increase mutation induction after EMS treatment. The p53 gene product did not have a major influence on the molecular spectrum after treatment with EMS, while folate deficiency increased the frequency of deletions in both cell types.

INTRODUCTION

Folate compounds are essential co-factors for purine and pyrimidine synthesis and for DNA methylation [1]. Consequently a deficiency of the vitamin, folic acid, is associated with a variety of genetic abnormalities. Chromosomal gaps and breaks, fragments, triradial and quadriradial forms, allocyclic chromosomes, micronucleus formation, increased rates of sister chromatid exchanges, expression of fragile sites and gene amplification have been described after nutritional deprivation of folic acid both *in vitro* and *in vivo* (reviewed in [2,3,4]). Measurements of mutant frequencies at the *aprt* and *hprt* locus in Chinese hamster ovary cells [5,6], and the *hprt* locus in rat splenocytes [7] and the human *HPRT* locus in peripheral blood lymphocytes indicate that nutritional folate deficiency is slightly mutagenic [8,9]. However, treatment of folate deficient CHO cells in culture or nutritionally depleted rats *in vivo* with alkylating agents results in a synergistic increase of mutant frequencies at the *hprt* locus [5,7].

The mechanism for this synergy is not completely understood. Several studies have demonstrated that folic acid deficiency alone results in decreased thymidylate synthesis, extensive incorporation of uracil into DNA, and strand breaks [4,6,10-15]. Repair of uracil residues and *N*-alkylation products utilize base excision repair [16,17]. However, defective DNA excision repair has been described in folate deficient CHO cells and rat colon [15,18,19]. Molecular studies in CHO cells from our laboratory found that treatment of folate deficient CHO cells with ethyl methanesulfonate (EMS), which causes a mixed S_N1/S_N2 type reaction, resulted in a large number of intragenic deletions and G>A mutations at non-CpG sites, particularly when these bases were flanked on both sides by G:C base pairs [20,21]. We postulated that these genetic changes were due to impaired ligation of single-strand breaks generated during base excision repair and a decreased capacity to remove O^6-ethylguanine. Taken together, these

investigations by our laboratory and by others support the following model: (1) folate deficiency causes extensive uracil incorporation into DNA and, (2) the greatly increase utilization of base excision repair to remove uracil and to correct alkylator damage leads to error-prone DNA repair [21].

The current studies were performed to test this model in human cells. The human lymphoblast cell line, TK6, was employed because it is near diploid with a stable karyotype (47, X,Y 13+), and it has been used extensively for studies of mutations at the *HPRT* locus [22,23]. Like CHO cells, TK6 cells are deficient in O^6-alkylguanine-DNA-alkyltransferase (AGT) [24-27]. Parallel studies were performed with the WTK1 lymphoblast cell line that was derived from the same donor as TK6 [22]. It has the same karyotype, identical growth and morphologic characteristics, and very similar doubling times as TK6, but differs from TK6 in that it has a homozygous mutation in codon 237 of exon 7 in the p53 gene, resulting in expression of mutant p53 protein and no wild-type p53 protein [22,23,28-29]. The gene product of p53 plays a role in excision repair [30-32] and since CHO cells are mutant at the p53 locus [33], it was of interest to compare the interactive effects of folate deficiency and alkylating agents on human cells that have wild-type and mutant p53 protein and contrast the results with our prior findings in CHO cells.

MATERIALS AND METHODS

Cell culture. The TK6 and WTK1 cell lines were obtained from Dr. Howard Liber [22]. Cells were grown in folate-free RPMI Medium 1640 (Gibco cat. #2716-021) or complete RPMI supplemented with 10% calf serum (Hyclone cat. #A-2151-1). For folate-free conditions, the cells were grown for 3 days in folate-free medium, and treated in folate-free medium before returning to complete medium. For EMS treatment and mutant isolation, the cells were spun

down and resuspended at 1 x 10^6 cells/ml in 6 identical 5 ml cultures. Five of these cultures were treated with 30 μg/ml EMS overnight. The cells were then spun down and washed twice. Survival plates were seeded from each of the original five cultures and the control. Each culture was then divided into 4 subcultures (giving 20 independent treated cultures) and grown in complete medium for 7-8 days to go through phenotypic lag. Cells from each of the 20 cultures were then plated in the presence and absence of 10 μM 6-thioguanine (TG) for mutant frequency determination. Two mutants were isolated from each TG selection plate and expanded. Samples of 1 x 10^4 cells from each of these mutant cultures were then snap frozen for mutational analysis and also stored in liquid nitrogen for subsequent growth if necessary.

The lymphoblast cloning assay was performed as previously described [34]. For determination of non-selected and TG selected CE, cells were seeded into 96-well microtiter plates (round bottom; Nunc) at 1-10 and 1-2 x 10^4 cells per well, respectively. At 10-14 days colony growth was determined using an inverted phase contrast microscope and the CE calculated by use of the Poisson relationship, $P_0 = e^{-x}$, where P_0 is the fraction of wells without cell growth. The CE(X) equals -ln P_0 /N, where N = the average number of cells/well. The mutant frequency is the ratio of the mean CE in the presence (selected) and absence (non-selected) of TG.

cDNA synthesis, PCR amplification, multiplex PCR, and DNA sequencing. Cell lysis and synthesis of cDNA was performed as described previously [36]. Amplification of the cDNA was done in two rounds of nested PCR in a Perkin-Elmer 2400 Thermal Cycler. A 5μl aliquot of cDNA was transferred into a first round mix of 1μl 25mM $MgCl_2$, 2μl 10x PE Buffer II (Perkin-Elmer), 0.5μl each 2.5mM dNTP's (Perkin-Elmer), 14.625μl HPLC water (Sigma), 0.125μl each 10 pM/μl forward (ssj) and reverse (rsj) primers (Table 1) (Gibco BRL Life Technologies, Grand

Island, NY) and 0.125µl Amplitaq polymerase (Perkin-Elmer) and amplified with a PCR profile of 94°C-5 minutes, 30 cycles of 94°C-1 minute, 65°C-1 minute, 72°C-2 minutes and a final extension of 72°C for 7 minutes. The second round amplification consisted of 3µl 25mM $MgCl_2$, 5µl 10 x PE Buffer II, 1µl each 2.5mM dNTP's, 36.25µl HPLC water, 0.25µl each 10 pM/µl forward (B) and reverse (4b) primers (Table 1), 0.25µl Amplitaq, and 1µl of the first round product and amplified with a PCR profile of 30 cycles of 94°C-1 minute, 55°C-1 minute, 72°C-2 minutes with a final extension of 72°C for 7 minutes. The final product was run on a 1% agarose (Gibco BRL Life Technologies, Grand Island, NY) gel, stained with ethidium bromide and observed under UV light. The product was excised, Gene Cleaned (Bio 101, Vista, CA) and sequenced with an ABI 373 sequencer (primers in Table 1).

Lysis for genomic PCR was carried out in a Thermolyne Amplitron II thermal cycler with a cycle profile of 56°C for 1 hour followed by 96°C for 10 minutes. A 10µl aliquot consisting of 40µl Tris-EDTA (TE), 2.5µl 10% Tween in TE, 2.5µl 10% NP-40 in TE and 5µl 1mg/ml proteinase K was added to the cell pellets and mixed well. Multiplex PCR was performed as described previously [35] with the following exceptions: a 5µl aliquot of lysed genomic DNA was added to the PCR mix and PCR was carried out with a cycle profile of 94°C for 5 minutes, 35 cycles of 94°C-1 minute, 59°C-1minute, 68°C-2 minutes, with a final extension of 68°C for 5 minutes in a Perkin-Elmer 2400 Thermal Cycler. The PCR product was run on a 2% agarose gel, stained with ethidium bromide and observed under UV light. The bands of interest were excised, Gene Cleaned and sequenced with an ABI 373 sequencer.

Deoxyuridine suppression tests. Deoxyuridine suppression tests were performed as previously described [37].

Statistical Analysis. Each group in the experiments relative CE vs. EMS and mutant frequency vs. EMS was entered into the General Linear Model as indicator variables while the EMS was entered along with interactions of EMS with the group indicator variables. This model was compared to the model that only included the indicator variables and EMS using an F-statistic. That is, we modeled the data using different slopes and modeled the data with equal slopes, and then compared the fit of the two models. A difference in the fit of the two models provided evidence that the slopes are different.

RESULTS

TK6 cells displayed a slightly more rapid doubling time (mean of 0.63 days, range 0.3-0.86 days, n=8) than WTK1 cells (mean of 0.8 days, range 0.64-1.1 days, n=6) in both folate-replete and deficient media. Growth slowed in both cell lines on Day 4 of culture in folate-free medium and the cells entered a quiescent phase with a relatively stable cell number. The addition of folic acid to the culture medium of cells on Day 8 led to a resumption of cell proliferation, indicating that the cells remained viable in the folate-free medium, as illustrated for WTK1 in Figure 1. However, metabolic folate deficiency, as measured by the deoxyuridine suppression test, was detectable by Day 3 and became more severe on Day 5 in both cell lines after incubation in folate-free medium (data not shown).

Treatment of the cell lines with increasing concentrations of EMS resulted in a progressive decline in CE. The range of 0 to 50 μg/ml was most informative. Figure 2 shows the results of 5 separate experiments in which TK6 and WTK1 cells were incubated for 3 days in folate-replete or deficient media and then treated with EMS in this concentration range. WTK1

cells were more resistant to the cytotoxic effects of EMS than TK6 cells in both folate replete and deficient media. Folate deficient TK6 and WTK1 cells were slightly more resistant to the toxic effects of EMS than folate replete cells. However, there was considerable variability among the experiments, and statistical analyses indicated that the slopes for the survival curves were similar.

The mutant frequencies at the *HPRT* locus determined from these 5 experiments are shown in Figure 3. The background mutant frequency was slightly higher in folate replete WTK1 cells than in TK6 (5.9 ± 4.6 vs. $3.3 \pm 2.8 \times 10^{-6}$, respectively [mean ± SD]), and rose somewhat higher in folate deficient medium ($13.9 \pm 11.6 \times 10^{-6}$ for WTK1; $4.5 \pm 3.1 \times 10^{-6}$ for TK6). None of these differences was significant by the unpaired t test. After treatment with EMS, the mutant frequencies were higher in TK6 than WTK1 cells in both folate replete and deficient medium. The mutant frequencies were higher in folate deficient than replete cells, but the effect was greater in TK6 cells. Statistical analysis of the slopes indicated significantly different *HPRT* mutation dose-response relationships ($P<0.01$).

To determine the molecular spectrum of the *HPRT* mutations, cDNA sequencing was initially performed followed by genomic multiplex PCR and/or sequencing as necessary to define the mutation. 159 mutant clones were analyzed. Seven pairs of mutants with identical mutations and arising in the same culture were found (mutations #9, 14, 63, 90, 106, 116 and 120). These pairs are believed to be non-independent and are considered as one mutation in the following analyses. Of the 152 mutations thus analyzed, 15 did not yield a cDNA after attempts on three different cell pellets. Of the 137 cDNAs sequenced, 37 showed exclusion of one or more complete exons, 9 showed partial exon exclusion and 8 showed inclusion of intron sequences. Of the 37 complete simple exon exclusion(s) mutants, 31 showed single base

substitution mutations in splice sequences, 2 mutants showed no change in the genomic region containing the exon and only 4 mutants were genomic deletions. Of the other 17 splice alteration mutations, 16 were single base substitutions and one contained a deletion. Of the 15 mutants which did not yield a cDNA, 11 showed deletion of *HPRT* exons in genomic DNA. These molecular analyses of the *HPRT* mutants are presented in detail in Table 2; the analyses are summarized in Table 3. The predominant mutation (95 of 152 = 63%) in both cell types grown in the presence or absence of folic acid was a G>A transition on the non-transcribed strand. A smaller number (21 of 152, 14%) of G>A transitions occurred on the transcribed strand. These transitions on the transcribed strand appeared to be more frequent in the folate-deficient WTK1 cells. These transitions were mainly (91 of 95) at non-CpG sites, particularly when these bases were flanked 3' by a purine or on both sides by G:C base pairs. The section of exon 3 that contains six consecutive guanines (207-212) was especially susceptible to mutation (19 of the 95 G>A mutations, 20%). The most striking difference between the folate-replete and deficient cells was an increased frequency of deletions and possible translocations in the cells of both types grown under low-folate conditions. In folate replete cells, 3 of 77 mutations (3.9%) were genomic deletions compared to 13 of 75 (17.3%) in folate deficient cells. In addition, 4 mutations in low folate cells resulted in no cDNA and no obvious change in the 9 *HPRT* exons and could represent translocation events, or deletions that interfered with RNA splicing. So the total deletion frequency could be as high as 17 of 75 (23.7%) in the low folate mutations.

Splice site mutations were common in both cell types and under both folate conditions, representing approximately 31% of mutations. Of the 50 mutations which affected splicing, 33 caused simple exon exclusions in the cDNA. The other 17 are presented in detail in Table 4. There were 9 partial exon exclusions (Table 4) (mutations #2, 19, 22, 36, 43, 59, 69, 81 and 92)

and 8 intron inclusions (mutation #1, 34, 35, 39, 40, 79, 80 and 95). There were two unusual intron inclusion splice effects. One mutant (#1, Table 4) showed the inclusion of 38 bases between exons 1 and 2 in cDNA. Inspection of the genomic sequence flanking these 38 bases revealed a reasonable splice acceptor and donor sequences, consistent with this inclusion being a cryptic exon. The reason for the use of these splice sequences is not yet known. We hypothesize that it is recognized as the result of a deletion or insertion in intron 1. Mutant #92 (Table 4) showed multiple cDNA products. The actual mutation is a G>A transition in the first base of the splice donor sequence (IVS3+1G>A). One cDNA results from the use of the new splice donor sequence GT_{318}atga resulting in the exclusion of the last two bases of exon 3 (317-318) and, in addition, the inclusion of 41 bases from the middle of intron 3. Inspection of these latter sequences flanking the inclusion reveals reasonable splice acceptor and donor sequences, defining it as another cryptic exon.

New splice sites were created in three mutations and cryptic splice sites were utilized because of mutations in the normal splice sequences in eight mutations. Most of these mutations have been reported previously [39]. Nearly all splice site mutations involved a G>A transition, often at a GT sequence (Table 4).

DISCUSSION

These studies indicate that the combination of nutritional folic acid deficiency and a monofunctional alkylating agent (EMS) causes similar genetic damage in human lymphoblast cell lines and CHO cells. Both the WTK1 lymphoblastoid cell line and CHO cells are derived from mammalian cells, and both are deficient in AGT and in p53 protein [22-29,33]. Therefore it is not surprising that the WTK1 and CHO cell lines react similarly to these DNA damaging

agents. The present studies extend and confirm our prior results in CHO cells and support the notion that they are relevant to human cells.

Treatment of WTK1 and TK6 cells with EMS resulted in dose-dependent cytotoxicity. However, inhibition of colony formation occurred at much lower concentrations than was seen previously with CHO cells. For example, the relative survival of CHO cells was about 40% with concentrations of EMS of 800 μg/ml, while the CE dropped to below that range with as little as 50 μg/ml of EMS in the lymphoblastoid cells treated under the same conditions [5]. Other laboratories have published similar relative sensitivities of these cell lines to EMS [23, 39]. In standard culture medium containing folic acid, WTK1 cells were more resistant to the cytotoxic effects of EMS than TK6 cells, confirming the previous report of Honma and colleagues [23]. The WTK1 cell line also has been shown to be more resistant than TK6 to X-ray induced cell killing [22,28]. Since p53 protein mediates apoptosis after DNA damage, a delay or partial abrogation of apoptosis in the p53-deficient WTK1 cell line may account for its superior survival after alkylating agent treatment. For example, apoptotic death is delayed for 3 days in WTK1 compared to TK6 after X-ray treatment [27]. Incubation of the lymphoblastoid cell lines in folate-deficient medium tended to increase their resistance to EMS cytotoxicity. Studies in other laboratories have shown that *in vitro* folate deficiency induces apoptosis in CHO cells, accumulation of human hepatoma HepG2 cells in S-phase and coincident apoptosis, and apoptosis of late-stage mouse erythroblasts [40-42]. It is therefore surprising that folate deficiency did not increase the sensitivity of the human lymphoblastoid cells to alkylating agent treatment.

The background mutant frequencies at the *HPRT* locus in the experiments reported here were slightly higher in WTK1 cells than in TK6. Amundson and colleagues found that the

formation of spontaneous *HPRT*$^-$ mutants was the same in WTK1 and TK6 [22], while Honma et al. reported an 8.3-fold higher spontaneous mutant frequency at the *HPRT* locus in WTK1 cells than in TK6 cells [23]. Our findings were intermediate between these two reports. Background mutant frequency increased modestly in both cell lines after incubation in folate-deficient medium, consistent with prior experiments in CHO cells wherein folate-deficient cells had slightly higher background mutant frequencies than folate-replete cells at the *hprt* [5] and *aprt* loci [6].

After treatment with EMS, TK6 cells had a higher mutant frequency than WTK1 at all drug concentrations tested. Amundson et al. found that *HPRT* mutant frequencies were slightly but not significantly higher in TK6 than in WTK1 after EMS treatment [22]. In contrast, X-ray induced mutant frequencies at the *HPRT* locus were higher in WTK1 than in TK6 [22], and mutant frequencies at another locus, thymidine kinase, also were higher in WTK1 than in TK6 after EMS or X-ray treatment [22,23,28]. The greater mutability of the autosomal thymidine kinase locus may be due to more frequent inter- and intramolecular recombination events [43]. Folate deficiency enhanced the mutagenesis of EMS in both TK6 and WTK1, consistent with our previous findings in CHO cells [5], but the effect was greater in the p53 positive TK6 cells.

The p53 gene product has been implicated in several DNA repair pathways. For example, p53 is involved in the induction of O^6-methylguanine-DNA methyltransferase through promoter activation after DNA damage [44]. The hMSH2 gene is a p53-regulated target gene, suggesting that p53 is also involved in DNA mismatch correction [45]. Wild-type p53 is required for global genomic nucleotide excision repair but not transcription-coupled repair [30,46], for base excision repair [32], and for ionizing radiation-induced modulation of excision repair [31]. DNA strand breaks associated with excision repair are potent inducers of p53 [47],

and p53 binds to ends and single-stranded gaps in DNA [48]. In addition, Lee and coworkers found that p53 recognized insertion/deletion mismatches [48]. Therefore, increased p53 activity has been associated with the types of DNA lesions previously described in folate-deficient cells treated with alkylating agents; namely, strand breaks, intragenic deletions, and single base mismatches. In the experiments reported here, we did not find that enhanced DNA repair mediated by p53 activity ameliorated either the cytotoxic or mutagenic activities of monofunctional alkylating agents in folate-deficient cells. Nutritional folate deficiency augmented genetic damage by EMS in the p53 mutant WTK1 cells, and, if anything, the augmentation was greater in folate-deficient TK6 cells that expressed wild-type p53.

As in CHO cells, the predominant mutation in EMS-treated folate-replete WTK1 cells was a G>A transition. EMS is mutagenic by reaction with the O^6 and N^7 positions of guanine. O^6-ethylguanine is mutagenic by pairing with thymine during replication, while N^7-alkylation products lead to apurinic sites that are processed by base excision repair and may cause mutations by defective repair or by mis-incorporation [49-53]. Under folate-replete conditions, most mutations (69%) in the WTKI cells were G>A transitions, suggesting that the deficiency of the AGT repair mechanism was a major contributing factor to persistent mutations. The smaller number of genomic deletions (8%) probably reflects error-prone base excision repair, because base excision repair defective cell lines exhibit increased percentages of deletion mutations after EMS treatment [54,55]. The mutational spectrum in the p53-competent TK6 cell line was similar to both CHO cells and WTK1 cells after EMS treatment in folate-containing medium, showing 76% G>A transitions but no deletions. This observation suggests that the p53 gene product does not have a major influence on the molecular spectrum after treatment with monofunctional alkylating agents.

Folate deficient TK6 and WTK1 cells, like folate replete cells, showed a predominance of G>A transitions. However they also exhibited an increased percentage of deletions compared to folate-replete cells. This finding supports and confirms our previous report that folate-deficient CHO cells had more intragenic deletions after EMS treatment than folate replete cells [20].

Folate deficient WTK1 cells were found to have a higher percentage of C>T transitions (21%) than either folate replete WTK1 cells (10%) or TK6 cells regardless of folate status (16% replete, 8% deficient). This higher percentage of C>T transitions may represent persistence of G>A transitions on the transcribed strand of these folate-deficient, p53 mutant cells. Alternatively, O^2-ethylcytidine may act as uracil and code for thymine [56] giving the pathway: O^2-ethylcytidine $\rightarrow$ U $\rightarrow$ T.

Treating all G/C$\rightarrow$A/T transition mutations, except the four that occur at CpG dinucleotides (two each of 151C$\rightarrow$T and 508C$\rightarrow$T), as the result of adducts on the G base, there are 112 G$\rightarrow$A mutations. Considering the flanking bases, 54% (61 of 112) occurred at Gs flanked by purines (21 at G<u>G</u>G, 17 at G<u>G</u>A, 14 at A<u>G</u>G and 9 at A<u>G</u>A), 22% (25 of 112) at Gs with a 5' purine (14 at A<u>G</u>T, 7 at G<u>G</u>T, 3 at A<u>G</u>C and 1 at G<u>G</u>C) and 13% (14 of 112) at Gs with a 3' purine (9 at T<u>G</u>G and 5 at T<u>G</u>A). Only 10% (12 of 112) occurred at Gs flanked by pyrimidines (8 at T<u>G</u>T and 4 at T<u>G</u>C). Thirty-six of these 112 G$\rightarrow$A mutations occurred in splice donor or acceptor sequences (32%). Twenty-eight of these 36 (78%) occurred at ag(a/g) (10) or (a/g)gt (18) sequences, reflecting the known importance of the acceptor <u>a</u>g dinucleotide and the donor gt dinucleotide [38]. Of the total of 23 A<u>G</u>(A/G) mutations, 10 occurred in splice sequences (10 of 36, 28%) compared to 13 in coding sequences (13 of 76, 17%). Of the total at 21 (A/G)<u>G</u>T mutations, 18 occurred in splice sequences (18 of 36, 50%) compared to 3 mutations in coding sequences (3 of 76, 4%). The most frequent mutation in splice sequences

was at AGT (10 of 36, 28%); mutations at AGT were found only rarely in the coding sequence (4 of 76, 5%).

The above result demonstrates the importance of defining a complete spectrum. Table 5 presents a summary of the cDNA phenotype and the actual mutation type. The 37 complete simple exon exclusion cDNAs consisted of 33 single base substitution mutations and only 4 genomic deletions. Eleven of the 16 genomic deletion mutations did not yield a cDNA. These results demonstrate the importance of not simply characterizing mutations only through cDNA sequencing. Genomic analysis is essential for differentiation of genomic deletion mutations. It was only through the latter analysis that the increase in genomic deletions resulting from treatment of cells in low folate medium was demonstrated, i.e. 4% (3 of 77) in folate replete vs. 17% (13 of 75) in low folate conditions.

Since the findings reported here occurred in human cell lines, they may be clinically relevant. Our results suggest that cells expressing p53 activity exhibit a higher rate of mutation induction but are more sensitive to the toxic effects of alkylating agents than those lacking p53 activity. Folate deficiency tends to reduce toxicity but increase mutation induction after alkylating agent treatment. TK6 cells are deficient in alkyltransferase activity and resemble in this regard bone marrow myeloid precursors [57]. The bone marrow is a frequent target organ for alkylating agent-induced transformation, and acute myelogenous leukemia has been reported following exposure to cyclophosphamide, melphalan and busulfan as single agents and to the combination of nitrogen mustard and procarbazine [58]. Cytogenetic analyses indicate that transformation to myelodysplasia and acute leukemia is associated with deletions and translocations [58]. Thus the combination of alkylating agent exposure and nutritional folate deficiency in a cell type deficient in alkyltransferase but expressing wild-type p53 creates an

environment that increases the risk of developing carcinogenic genetic changes. The addition of a mutation at the p53 locus would alter further the biology of a developing tumor cell, in that loss of p53 activity reduces toxicity from monofunctional alkylating agents, and those cells lacking p53 activity and folate are most resistant. The corollary of these observations is that correction of nutritional folate deficiency may reduce the risk of developing carcinogenic genetic changes in normal cells after alkylating agent exposure and enhance the sensitivity to alkylating agents in p53-mutant malignant cells. The latter interpretation may explain, at least in part, our observation that mammary carcinoma was more resistant to cyclophosphamide chemotherapy in folate deficient rats than in folate replete animals [59].

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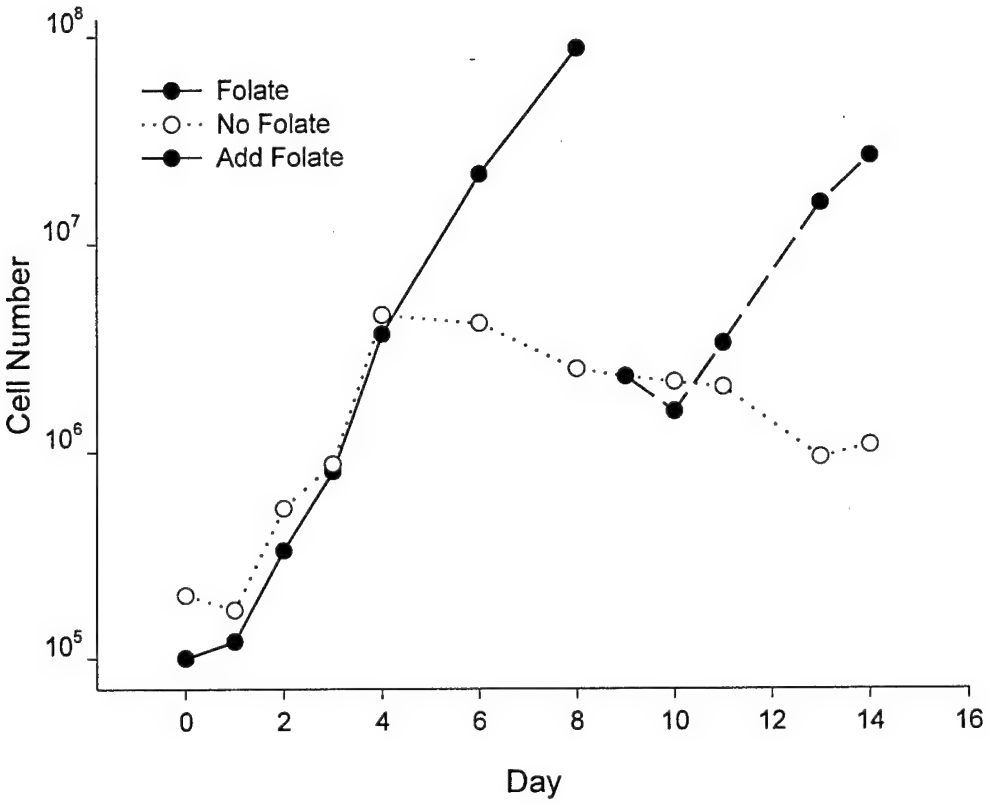
Folate
No Folate
Add Folate
Cell Number
10^8
10^7
10^6
10^5
0
2
4
6
8
10
12
14
16
Day

Figure 1

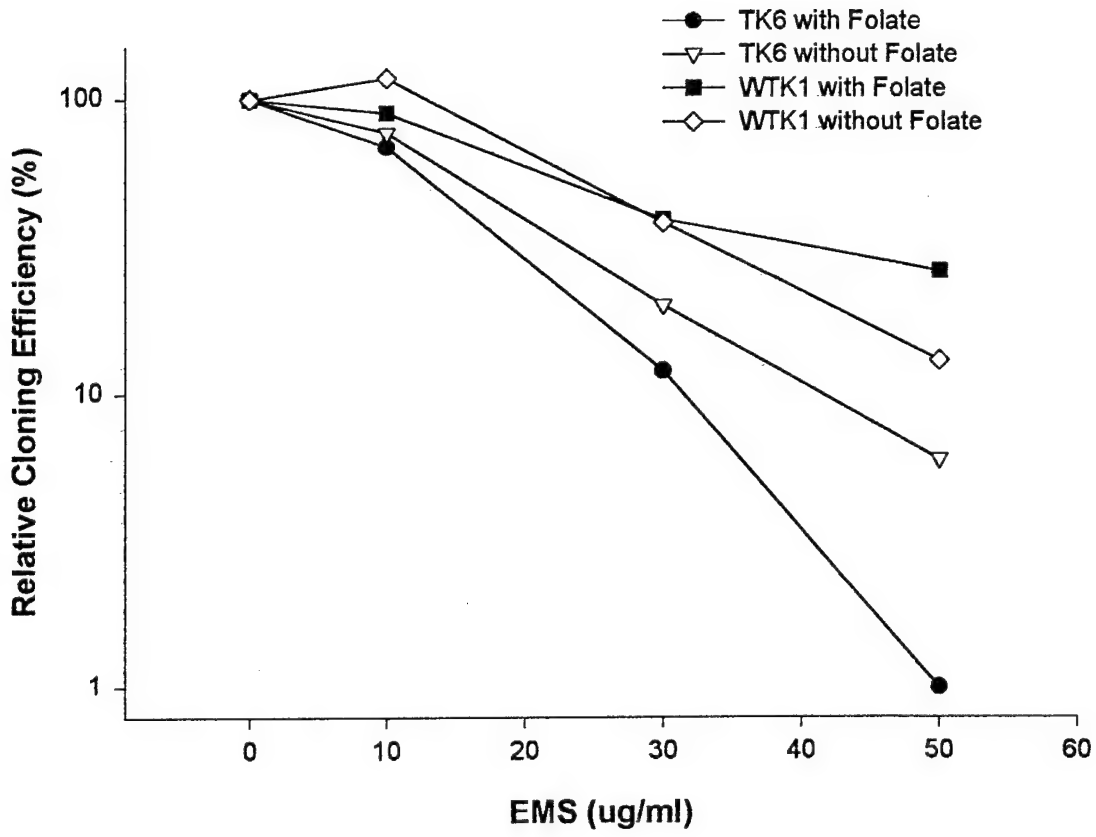
TK6 with Folate
TK6 without Folate
WTK1 with Folate
WTK1 without Folate
Relative Cloning Efficiency (%)
100
10
1
0
10
20
30
40
50
60
EMS (ug/ml)

Figure 2

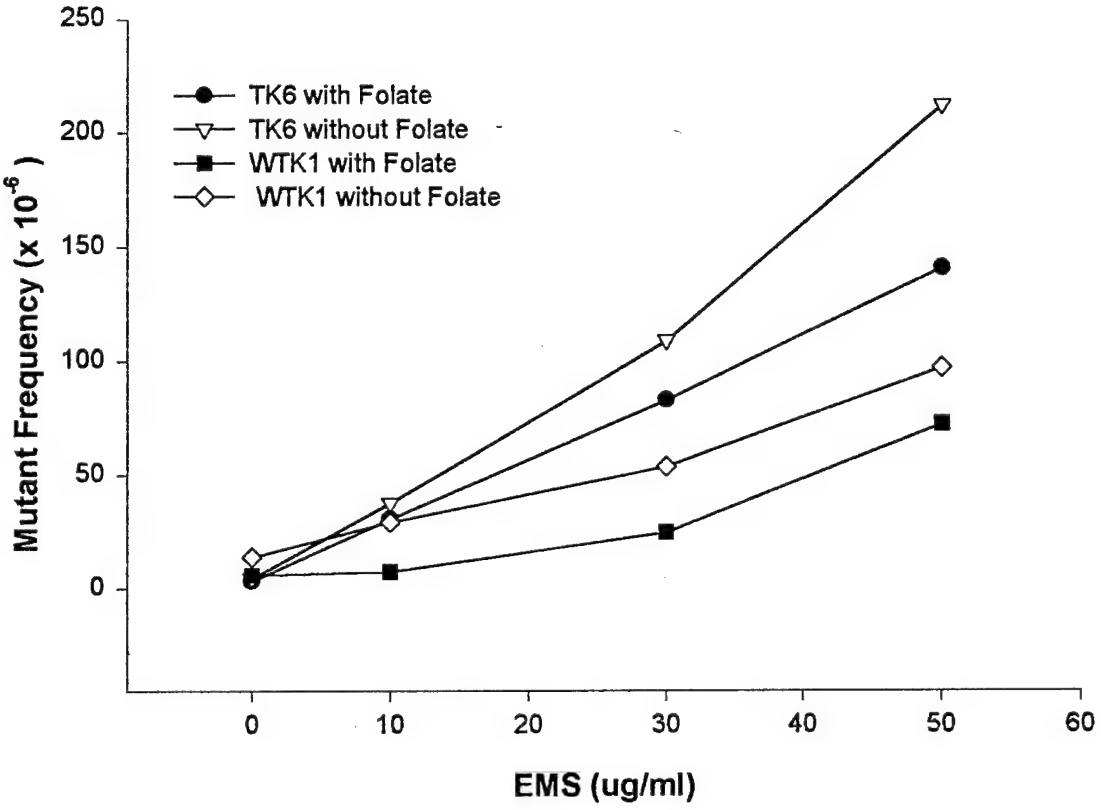
250
200
150
100
50
0
Mutant Frequency (x 10^{-6})
TK6 with Folate
TK6 without Folate
WTK1 with Folate
WTK1 without Folate
0
10
20
30
40
50
60
EMS (ug/ml)

Figure 3

LEGENDS

Figure 1. Proliferation of WTK1 human lymphoblastoid cells in folate replete and deficient culture media. Cells in folate replete medium proliferated with a doubling time of about 0.8 days (closed circles, solid line). Cells in folate deficient medium proliferated at approximately the same rate until day 4, when they entered a quiescent phase (open circles). Addition of folic acid to the culture medium of these cells on Day 8 led to a resumption of proliferation at the prior rate (closed circles, broken line), while cells remaining in folate deficient medium did not increase in number.

Figure 2. Effect of folate status and EMS treatment on relative cloning efficiency of human lymphoblastoid cells. Cells were cultured in media containing or lacking folic acid. They were treated with EMS in the indicated concentrations, and cloning efficiency was determined relative to untreated cells. Each point represents the mean of 5 separate experiments.

Figure 3. Mutant frequency at the *HPRT* locus in human lymphoblastoid cells after treatment with EMS. Each point represents the mean of 5 separate experiments.

Table 1

Oligonucleotide primers used for RT-PCR and sequence analysis of *HPRT* mutations.

Name	Sequence	Base Numbers
PCR primers		
ssj (sense)	cctctgctccgccaccg	1614 to 1630
rsj (antisense)	cgcccaaagggaactgatagtctataggc	41719 to 41691
B (sense)	cctgagcagtcagcccgcgc	1641 to 1660
4b (antisense)	gcaaaaagctctactaagcagatggccacag	41608 to 41578
Sequence primers		
B (sense)	cctgagcagtcagcccgcgc	1641 to 1660
A (antisense)	tcaataggactccagatgttt	41546 to 41526

Base numbers listed are 5' to 3' for the *HPRT* genomic sequence [Genbank]

Table 2

Mutation #[a]	Mutant code	cDNA alteration (exon in which mutation is located)	Genomic alteration	Sequence context	Amino Acid Change
A. Folate Replete TK6 p53+					
1	LT2-5D1	Intron inclusion 9192-9230[b]			Insertion of 13 aa between exon 1 and exon 2
2	LT2-3D2	Exclusion 28-32 (Exon 2)	IVS1-1G>A	ttt cag ATT	TGA STOP at new codon 10
3	LT2-5D2	148G>A (Exon 3)		CTT GCT CGA	50ala>thr
4	LT2-1B2	208G>A (Exon 3)		AAG GGG GGC	70gly>arg
5	LT2-1D2	208G>A (Exon 3)		AAG GGG GGC	70gly>arg
6	LT2-4B1	208G>A (Exon 3)		AAG GGG GGC	70gly>arg
7	LT2-1C1	209G>A (Exon 3)		AAG GGG GGC	70gly>glu
8	LT2-4D2	209G>A (Exon 3)		AAG GGG GGC	70gly>glu
9	LT2-5A1 & 5A2	209G>A (Exon 3)		AAG GGG GGC	70gly>glu
10	LT2-5B1	209G>A (Exon 3)		AAG GGG GGC	70gly>glu
11	LT2-1C2	Exon 4 Exclusion	IVS3-1G>A	aac tag AAT	Inframe loss of exon 4 (aa107-128)
12	LT2-2A1	Exon 4 Exclusion	IVS3-1G>A	aac tag AAT	Inframe loss of exon 4 (aa107-128)
13	LT2-1B1	Exon 4 Exclusion	IVS4+1G>A	AAG gta tgt	Inframe loss of exon 4 (aa107-128)
14	LT2-3A1 & 3A2	Exon 5 Exclusion	IVS4-1G>A	ttc tag AAT	Inframe loss of exon 5 (aa129-146)
15	LT2-2B1	400G>A (Exon 5)		GTG GAA gta	134glu>lys

68	LT3-5A1	Exon 7 Exclusion	IVS7+5G>A	Tgt aag tga	TGA STOP at new codon 166
69	LT3-5A2	Exclusion 533-553 (Exon 8)	IVS7-1G>A	tta gTT GTT	Inframe loss of 1st 8 aa of exon 8 and 178phe>tyr
70	LT3-2A1	539G>A (Exon 8)		GTT GGA TTT	180gly>glu
71	LT3-2B2	539G>A (Exon 8)		GTT GGA TTT	180gly>glu
72	LT3-2D1	539G>A (Exon 8)		GTT GGA TTT	180gly>glu
73	LT3-3C1	539G>A (Exon 8)		GTT GGA TTT	180gly>glu
74	LT3-1D1	601G>A (Exon 8)		AGG GAT TTG	201asp>asn
75	LT3-5D1	635G>A (Exon 9)		ACT GGA AAA	212gly>glu
76	LT3-4B2	no cDNA	del Exon 1 thru 9		No protein made
77	LT3-1D2	no cDNA	del Exon 4 thru 9		No protein made

C. Low Folate TK6 p53+

78	LT1-4D1	2T>G (Exon 1)		gtt ATG GCG	1met>arg
79	LT1-4C2	27G>A and Inclusion IVS1+1 to+49		GTC GTG gtg	TAG STOP at new codon 27
80	LT1-4A2	IVS1+1G>A and Inclusion IVS1+1 to +49		GTG gtg agc	TAG STOP at new codon 27
81	LT1-5C1	Mix exon 2 Exclusion and Exclusion 28-32	IVS1-1G>A	ttt cag ATT	Immediate TGA STOP at new codon 10 OR TGA STOP at new codon 11
82	LT1-1A1	119G>A (Exon 2)		CAT GGA CTA	40gly>glu

83	LT1-1D2	134G>A (Exon 2)		GAC AGg taa	45arg>lys
84	LT1-4C1	134G>A (Exon 2)		GAC AGg taa	45arg>lys
85	LT1-1C2	Exon 2 Exclusion	IVS2+1G>C	GAC AGg taa	TGA STOP at new codon 11
86	LT1-2A1	Exons 2&3 Exclusion	IVS3+1G>A	TGT gtg agt	Inframe loss of exons 2&3 (aa10-106)
87	LT1-1B1	152G>C (Exon 3)		GCT CGA GAT	51arg>pro
88	LT1-4B2	208G>A (Exon 3)		AAG GGG GGC	70gly>arg
89	LT1-2C1	209G>A (Exon 3)		AAG GGG GGC	70gly>glu
90	LT1-3B1 & 3B2	209G>A (Exon 3)		AAG GGG GGC	70gly>glu
91	LT1-4A1	211G>T (Exon 3)		GGG GGC TAT	71gly>cys
92	LT1-5B2	Exclusion 317-318, exons 2&3 exclusion, exon 3 exclusion with Inclusion 26894-26934[b]	IVS3+1G>A	TGT gtg agt	Immediate TAA STOP at codon 106 OR Inframe loss of exons 2&3 (aa10-106) OR TAA STOP at new codon 47
93	LT1-2C2	Exon 4 Exclusion	IVS3-1G>A	aac tag AAT	Inframe loss of exon 4 (aa107-128)
94	LT1-5B1	355G>A (Exon 4)		GGT GGA GAT	119gly>arg
95	LT1-1A2	IVS5+1G>A and Inclusion IVS5+1-67		GAA gta agt	TAA STOP at new codon 136
96	LT1-2B1	+/- Exon 6 Exclusion	IVS5-2A>T	tga aag GAT	Normal or TAA STOP at new codon 147
97	LT1-2A2	425C>T (Exon 6)		AAA ACA ATG	142thr>ile
98	LT1-5D1	Ambiguous	430C>T	ATG CAG ACT	144gln>STOP
99	LT1-1B2	+/- Exon 8 Exclusion	538G>A	GTT GGA TTT	180gly>arg
100	LT1-3A2	+/- Exon 8 Exclusion	577C>T	GCC CTT GAC	193leu>phe

101	LT1-3A1	+/- Exon 8 Exclusion	589G>T	AAT GAA TAC	197glu>STOP
102	LT1-1D1	568G>A (Exon 8)		GTA GGA TAT	190gly>arg
103	LT1-5A2	568G>A (Exon 8)		GTA GGA TAT	190gly>arg
104	LT1-1C1	599G>A (Exon 8)		TTC AGG GAT	200arg>lys
105	LT1-4D2	599G>A (Exon 8)		TTC AGG GAT	200arg>lys
106	LT1-3C1 & 3C2	601G>A (Exon 8)		AGG GAT TTG	201asp>asn
107	LT1-3D1	626G>A (Exon 9)		ATT AGT GAA	209ser>asn
108	LT1-2B2	no cDNA	del Exon 1		No ATG START codon
109	LT1-2D2	no cDNA	del Exon 6, 7/8, 9		No protein
110	LT1-5A1	no cDNA	del Exon 1 thru 9		No protein
111	LT1-2D1	no cDNA	no change		
112	LT1-4B1	no cDNA	no change		
113	LT1-5C2	no cDNA	no change		
114	LT1-5D2	no cDNA	no change		

D. Low Folate WTK1 p53-

115	LT4-2D2	130G>T (Exon 2)		ATG GAC AGg	44asp>tyr
116	LT4-2B1 & 2B2	Exon 2 Exclusion	IVS2+1G>A	GAC AGg taa	TGA STOP at new codon 11
117	LT4-5B1	Exon 2 Exclusion	IVS2+1G>A	GAC AGg taa	TGA STOP at new codon 11
118	LT4-5D1	Exon 2 Exclusion	IVS2+1G>A	GAC AGg taa	TGA STOP at new codon 11

119	LT4-4B2	145C>T (Exon 3)		CGT CTT GCT	49leu>phe
120	LT4-4D1 & 4D2	151C>T (Exon 3)		GCT CGA GAT	51arg-stop
121	LT4-1B2	208G>A (Exon 3)		AAG GGG GGC	70gly>arg
122	LT4-1A1	209G>A (Exon 3)		AAG GGG GGC	70gly>glu
123	LT4-4C2	209G>A (Exon 3)		AAG GGG GGC	70gly>glu
124	LT4-4B1	Exons 2&3 Exclusion	IVS3+1G>A	TGT gtg agt	Inframe loss of exons 2&3 (aa10-106)
125	LT4-5A1	325C>T (Exon 4) ± Exon 2&3 Exclusion		GAC CAG TCA	109gln>stop
126	LT4-3D2	355G>A (Exon 4)		GGT GGA GAT	119gly>arg
127	LT4-2A2	400G>A (Exon 5)		GTG GAA gta	134glu>lys
128	LT4-5A2	400G>A (Exon 5)		GTG GAA gta	134glu>lys
129	LT4-3A1	416C>T (Exon 6)		GAC ACT GGC	139thr>ile
130	LT4-3A2	419G>A (Exon 6)		ACT GGC AAA	140gly>asp
131	LT4-1C2	430C>T (Exon 6) ± Exons 2&3 Exclusion		ATG CAG ACT	144gln-stop
132	LT4-3B2	464C>T (Exon 6)		AAT CCA AAG	155pro>leu
133	LT4-5D2	464C>T (Exon 6)		ATT CCA AAG	155pro>leu
134	LT4-3D1	508C>T (Exon 7)		CCA CGA AGT	170arg>stop
135	LT4-2A1	Exon 7 Exclusion	IVS7+1G>A	GAC Tgt aag	TGA STOP at new codon 165
136	LT4-2C2	Exon 7 Exclusion	IVS7+5G>A	Tgt aag tga	TGA STOP at new codon 165

Table 3

Summary of mutation types in the *HPRT* gene in 6-thioguanine resistant human lymphoblastoid cells exposed to EMS

Mutation Type		Folate Replete		Low Folate	
(No. of mutations)	NTS	TK6 (38)	WTK1 (39)	TK6 (37)	WTK1 (38)
Transitions					
GC>AT	G>A	29 (13)[a]	27 (10)[a]	21 (8)[a]	18 (7)[a]
	C>T	6 (1)	4	3 (1)	8
AT>GC	A>G	0	1	0	0
	T>C	0	1	0	0
Transversions					
GC>TA	G>T	0	2 (2)	2 (1)	1
	C>A	1	0	0	0
GC>CG	G>C	0	0	2 (1)[a]	0
	C>G	0	0	0	0
AT>TA	A>T	0	0	1 (1)[a]	1 (1)[a]
	T>A	0	0	0	0
AT>CG	A>C	0	0	0	0
	T>G	0	0	1	0
Genomic deletion		0	3	3	10
"new exon"		1	0	0	0
No change		1[b]	1[c]	4[d]	0

NTS = nontranscribed strand

[a]mutations which affected splicing

[b]exon 8 exclusion in cDNA with no change in genomic found

[c]exon 4 exclusion in cDNA with no change in genomic found

[d]four mutations with all nine exons present in genomic DNA and no change in the sequence of exons 1, 7, 8 and 9.

Table 4

HPRT splice alterations in 6-thioguanine resistant human lymphoblastoid cells exposed to EMS[a]

Mutation #	Mutant code	Alteration	Sequence context	Comments
A. Insertion of cryptic exons				
1	LT2-5D1	Intron inclusion 9192-9230	ttttttttttttttag|A_{9192}C(A)GGAGTCTCGCTCT GTCACTCAGGCTGGAGTGTAGTG$_{9230}$|gt atga	Analysis of the genomic region around the inserted bases indicates that there is an apparent acceptor just 5' and an apparent donor 3'. Thus, these bases form a reasonable although somewhat small exon (38bp). The area around exon 1 (b1651-1841) and the inserted bases (b9014-9546) was sequenced and no alterations were found. We speculate a large deletion or insertion elsewhere in intron 1 alters the splicing pattern and causes utilization of this new "exon." NOTE: There appears to be an error or polymorphism in the GeneBank sequence as A_{9194} was not present in our wild type sequence.

92	LT1-5B2	IVS3+1G>A	gctttatttttacatttag|A_{26894}TTTTAATCCGTCT GGAATTTATTTTTGTGTATGCTGTGAG$_2$ $_{6934}$|gtaggg	Multiple cDNA products were found: (1) exclusion of b317-318 with the inclusion of b26894-26934 (2) exclusion exon 3 with the inclusion b26894-26934 and (3) exclusion exons 2&3. Exclusion 317-318 GT was due to utilization of a new created splice sequence TT_{316}|GT_{318}atga. The sequences 5' and 3' of the included 26894-26934 appears to be an acceptor and donor, respectively. Because of the mutation of the exon 3 donor this 26894-26934 is processed as an alternative or additional "exon".

B. Creation of new splice sites

59	LT3-5C1	209G>T	AG_{207}|GT_{209}GGGC...$TATTGT_{318}$|gtgagt	Two cDNA products were found: (1) Loss of bases 208-318 in exon 3 due to creation of a new splice donor site at b208 and (2) and normal length cDNA with 209G>T (gly>val).

19	LT2-4A1	IVS5-1G>A	aaaa$\underline{a}_{IVS5-1}G_{403}$|ATA...$AAG_{485}$| aaaa$\underline{a}_{IVS5-1}G_{403}$ATA...$CAG_{452}$|GCA...$AAG_{485}$|	Splice acceptor site mutation that causes the exclusion of b 403 (the first base of exon 6) utilizing a new acceptor created one base shifted, or the exclusion of b 403-452 utilizing a cryptic acceptor at 452.
34	LT2-2B2	IVS8-16G>A	ct$\underline{a}$g|$a_{IVS8-14}$ttttttttatag$_{IVS8-1}$CATGTT	Inclusion of intron 8, b-1 to b-14 due to use of a new splice site (tag$_{IVS-15}$|a). This results in a chain terminating codon TAG at new codon 251 creating a larger protein product of 255 amino acids since the normal TAA at codon 251 is out of frame.
35	LT2-3C1	IVS8-16G>A		

C. Use of cryptic splice sites

79	LT1-4C2	27G>A	GT$\underline{A}g_{IVS1+1}$tgagc...cggcag$_{IVS1+49}$|gtggcg	Splice donor site mutations that cause the use of a cryptic splice site at IVS1+49. This 49bp inclusion is out of frame and yields a chain termination codon TAG at new codon number 27.
80	LT1-4A2	IVS1+1G>A	GTG$\underline{a}_{IVS1+1}$tgagc...cggcag$_{IVS1+49}$|gtggcg	
40	LT3-4A2	IVS1+5G>T	GTG$\underline{t}_{IVS1+1}$tgagc...cggcag$_{IVS1+49}$|gtggcg	

39	LT3-4C2	del 1655 to 1730	gc[$c_{IVS0-22}$cg...$gg_{IVS1+27}$]cc...$ag_{IVS1+49}$|gtggcg	Genomic deletion of 22 bases 5' to exon 1, exon 1, and 27 bases of intron 1 including the exon 1 splice site which causes the use of the cryptic splice site at IVS1+49 (gtggcg) to splice to exon 2.
2 43 81	LT2-3D2 LT3-3B2 LT1-5C1	IVS1-1G>A	ctttttcaa$_{IVS1-1}$$A_{28}$TTAG$_{32}$|GTG	Splice acceptor site mutation that results in 2 cDNAs: exclusion of exon 2 or the exclusion of bases 28-32 utilizing a cryptic acceptor at b32. This results in an immediate chain terminating TGA at new codon 10.
95	LT1-1A2	IVS5+1G>A	GAAa$_{IVS5+1}$taagt...aaag$_{IVS5+67}$|gtaagc	Splice donor site mutation that causes the use of a cryptic splice site at IVS5+67 which splices in 67 bases of intron 5. This inclusion results in a chain termination codon, TAA, at new codon 136.
22 69	LT2-3D1 LT3-5A2	IVS7-1G>A	ttaa$_{IVS7-1}$$T_{533}$TGTT...CCAG$_{553}$|ACAAG	Splice acceptor site mutation that creates mixed cDNA: (1) exon 8 exclusion and (2) b 533-553

				exclusion because of the use of a cryptic acceptor site at b553. The latter yields an inframe mRNA which predicts a protein lacking 7 amino acids.
36	LT2-4A2	IVS8-1G>A	ataa$\underline{}_{IVS8-1}C_{610}$ATG...TTAG$_{626}$|TGAA	Splice acceptor site mutation that causes the exclusion of b 610-626 in exon 9 due to the use of cryptic splice site TAG$_{626}$|T. This results in an immediate chain terminating TGA at new codon 204.

[a]does not include 33 simple exon exclusions listed in Table 4

Table 5

Summary of cDNA phenotype mutation type

	Folate Replete		Low Folate		Total
	TK6	WTK1	TK6	WTK1	
No. of Mutants	38	39	37	38	152
cDNA Positive	38	37	30	32	137
"Normal" Length	22	23	18	20	82
Complete Simple Exon Exclusion	9	9	7	12	37
Splice Mutation	9	9	7	8	33
Genomic Deletion	0	0	0	4	4
Partial Exon Exclusion	4	3	2	0	9
Splice Mutation	4	3	2	0	9
Intron Inclusion	3	2	3	0	8
Splice Mutation	3	1	3	0	7
Genomic Deletion	0	1	0	0	1
cDNA Negative	0	2	7	6	15
Genomic Deletion	0	2	3	6	11
Uncharacterized	0	0	4	0	4